THE UNIVERSITY OF ALBERTA

Fate of *Salmonella typhimurium* at suboptimal growth temperatures

by

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Willis M. Fedio

A THESIS

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IN

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To Gail, Sophie and Coco

ABSTRACT

The effects of suboptimal non-freezing temperatures on *Salmonella typhimurium* ATCC 13311 when stored in trypticase soy broth (TSB), 10% TSB or in a chicken meat system were investigated. Injury and death of *S. typhimurium* during chilled storage were examined using a differential plating technique. The physiological state of the cells, composition of the storage medium, temperature and time of storage were all shown to influence viability and injury of the cells. Exponential phase cells were more sensitive to chilling than stationary phase cells in both TSB and 10% TSB. Injury and loss of viability was greater in 10% TSB with its low cryoprotective capacity. No injury or loss of viability occurred when the organisms in either growth phase were inoculated onto chicken meat. Cells stored at 4°C did not grow, but, depending upon the physiological age and storage menstruum, cells were either injured and died (log phase cells in TSB and 10% TSB), or injured without loss of viability (stationary phase cells in 10% TSB), or survived without adverse effect (on chicken and stationary phase cells in TSB). Cells inoculated onto chicken and stored at 7°C grew, but during storage in the broths there was decreased viability and some injury. At 7°C under all conditions of storage, cell elongation to form filaments was observed. However this was interpreted as a form of abnormal cell growth rather than cell injury.

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1. INTRODUCTION

Salmonella spp. are well known agents of gastro-intestinal disease in humans. Foods of animal origin such as poultry, meat, eggs, milk and dairy products are most often the vehicles responsible for transmission of *Salmonella* spp. to humans (Hobbs, 1974; Silliker, 1980). *Salmonella* is the most significant pathogen transmitted by raw poultry to humans (Todd, 1980; Kampelmacher, 1983). Worldwide, poultry is an important food item and production is increasing to satisfy demand (Todd, 1980). Increased consumption of poultry has resulted in increases in poultry-associated foodborne disease, particularly salmonellosis (Todd, 1980; Kampelmacher, 1983).

Food animals can become contaminated with salmonellae on the farm, during transportation to slaughterhouses or during processing. In nature, the intestinal tracts of animals represent a major source of *Salmonella*. Salmonellae can infect poultry from contact in hatcheries, the farm environment and feed and water (Bryan *et al.*, 1979). While being transported to slaughterhouses in close proximity to one another, they contaminate each other and the transportation crates. In many cases, the stress related to transportation is known to cause salmonellae to be excreted by birds that otherwise were not excreting the organisms. Salmonellae are also transferred from one bird to another during defeathering (Bryan *et al.*, 1979).
Cross-contamination is known to occur during chilling,

weighing, packaging and further processing (Bryan *et al.*, 1979). Invariably, a large proportion of product coming out of poultry processing plants is contaminated.

Raw chicken is an important source of salmonellae, that contaminates the hands of workers, equipment and other foods. It has been suggested that raw and cooked poultry should be separated in time and space to avoid such cross-contamination (Bryan *et al.*, 1979; Todd, 1980). *S. typhimurium* is the most common serotype associated with chicken processing plants (Campbell *et al.*, 1983), and is the most frequent agent of *Salmonella* gastroenteritis in man (Krieg, 1984). The type strain of *S. typhimurium* is ATCC 13311 (Krieg, 1984) and this was used as the test organism throughout this study. Most of the salmonellae that are present on chicken may be in an injured state from the processing conditions. The objective of this study was to examine the fate of *Salmonella* that has been exposed to chilling or to refrigeration temperatures, in a way that simulates conditions associated with poultry and poultry processing.

2. LITERATURE REVIEW

2.1 Effect of Low Temperature on Bacterial Growth

2.1.1 Introduction

All microorganisms have a minimum, optimum and maximum growth temperature. These are known as the cardinal temperatures for growth. These temperatures vary widely depending upon the microorganism. Based upon their temperature ranges for growth, four major physiological groups of microorganisms have been distinguished by Olson and Nottingham (1980) as:

1. thermophiles - those with an optimum temperature for growth *ca.* 60°C, and a maximum for some as high as 75 to 90°C and a minimum about 35°C.
2. mesophiles - those with an optimum temperature between 30 and 45°C, with a maximum growth temperature below 50°C and a minimum growth temperature ranging from about 5 to 10°C.
3. psychrophiles - organisms having an optimum growth temperature of about 15°C, with a maximum temperature of about 20°C or lower and a minimum growth temperature at 0°C or lower.
4. psychrotrophs - organisms capable of growing at *ca.* 0°C, but having optimum and maximum growth temperatures similar to mesophiles.

Most attention has been placed on growth at the optimum

growth temperature. This review will concentrate on the effects of suboptimal temperatures on bacteria.

2.1.2 Effects of Suboptimal Temperatures

Bacterial growth can be defined as an increase in the quantity of cellular constituents and structures of an organism (Brock, 1979). Growth in the absence of cell division usually results in an increase in the size and weight of the cell. In most microorganisms, growth is followed by cell division resulting in an increase in cell number (Brock, 1979).

Growth of bacteria is the result of a complex and highly regulated series of chemical reactions. The effect of temperature on the rate of growth may be due to its effect on the velocity of chemical reactions (Ingraham *et al.*, 1983). Growth rate can be expressed in terms of the Arrhenius equation:

$$V = S.e^{-\Delta E/RT}$$

where V = velocity of the reaction

S = constant

ΔE = activation energy

R = gas constant

T = temperature in °K

By taking the logarithm of this equation,

$$\ln V = (\Delta E/R)(1/T)+\text{constant}$$

a linear relationship exists between reaction velocity and the reciprocal of the absolute temperature. The linear portion of the curve indicates the obedience of the growth rate to temperature on the Arrhenius relationship regarding chemical reactions. However, when applied to microbial growth the curve is linear only over a relatively narrow range of temperatures. At temperatures below this range a deviation from the linearity is observed. The curve finally becomes vertical indicating that growth has ceased (minimum growth temperature). Likewise, a decrease in growth rate is observed at temperatures above the optimum until growth terminates at the maximum growth temperature (Inniss and Ingraham, 1978).

At temperatures a few degrees above their minimum growth temperature, the growth rate of bacteria is usually slow and growth is often preceded by a long lag period (Matches and Liston, 1968b; Mackey *et al.*, 1980b). The minimum growth temperature can be regarded as the point where either the lag or generation time becomes infinite (Ingram and Mackey, 1976). The minimum temperature for growth can be defined as the temperature below which sustained bacterial growth does not occur (Shaw *et al.*, 1971). In most organisms, many independent biochemical functions stop simultaneously at the minimum growth

temperature (Ingraham *et al.*, 1983). This makes the biochemical analysis of low temperature inhibition of growth very difficult.

The likelihood that many vital functions cease simultaneously is supported by the observation that for most bacteria, mutants with decreased minimum growth temperatures cannot be isolated (Inniss and Ingraham, 1978). However, mutants of *Pseudomonas aeruginosa* with a decreased minimum growth temperature were induced using ultraviolet irradiation (Azuma *et al.*, 1962). Olsen and Metcalf (1968) repeated this study and found psychrophilic mutants with minimum and maximum growth temperatures changed from approximately 11 and 44°C to 0 and 32°C. There was a proportional lowering of the maximum growth temperature with the acquisition of ability to grow near 0°C. Using a generalized transducing phage, the researchers were able to transfer the psychrophilic character from *Pseudomonas fluorescens* to *P. aeruginosa*. They concluded that the delineation of growth temperatures of mesophilic bacteria may represent the products of a limited number of genetic loci, the primary function of which is regulation of cell division in response to temperature change. Since many of the pseudomonads are psychrophilic in nature, the genetic changes necessary to enable these organisms to grow at low temperatures might be expected to be low (Inniss and Ingraham, 1978).

Matches and Liston (1968b) stated that the minimum temperatures for growth of seven *Salmonella* serotypes determined by visible growth on agar ranged from 5.5 to 6.8°C. In broth culture, after 19 days incubation, the minimum growth temperatures for *Salmonella heidelberg*, *Salmonella typhimurium* and *Salmonella derby* were 5.3, 6.2 and 6.9°C, respectively.

The minimum growth temperature of a microorganism can be affected by environmental factors such as nutrient status, water activity and pH of the growth substrate (Ingram and Mackey, 1976). The minimum growth temperature of a microorganism is thought to be lower if suitable nutrients are present in the growth substrate (Ingram and Mackey, 1976). *Clostridium botulinum* type E is able to grow at 3.3°C in beef stew but only as low as 8°C in peptone water (Schmidt *et al.*, 1961, cited in Ingram and Mackey, 1976). The minimum growth temperature of *Yersinia enterocolitica* is 0°C on raw pork, 1.4°C in tryptic soy broth (TSB) and 4.5°C in a glucose-salts medium (Mungal, 1979). Thus, the lower temperature limit for growth of bacteria can be influenced by the nutritive composition of the growth substrate.

The acidity of the growth medium affects the minimum temperature for growth. Generally it is believed that a suboptimal pH leads to a higher minimum growth temperature (Ingram and Mackey, 1976). Matches and Liston (1972b) found that *S. typhimurium* would not grow at pH 5.0. At pH 6.0 and 7.0, the minimum growth temperature was 7.2°C, while at pH 8

and 9 the minimum growth temperatures were 6.4 and 8.0°C, respectively. Mungal (1979) found that the minimum growth temperature of *Y. enterocolitica* in TSB at pH 7 and 6 was 1.4 and 4.0°C, respectively. Thus, there appears to be a narrowing of the pH range permitting growth near the minimum temperature for growth.

The minimum temperature for growth is also influenced by the water activity (a_w) of the growth medium (Ingram and Mackey, 1976). Water activity (a_w) affects the minimum growth temperature of *S. heidelberg*, *S. derby* and *S. typhimurium* (Matches and Liston, 1972a). As the solute concentration of the growth medium increases, the water activity decreases. With a decreased a_w the lag phase of the organism increases and the rate of growth decreases (Matches and Liston, 1972a). At 8°C, growth of *S. typhimurium* was possible in nutrient broth containing 1% NaCl but not in broths containing 2, 3 or 4% NaCl. At 12°C, growth was possible in broth containing up to 4.5% NaCl and at 37°C up to 7% NaCl (Matches and Liston, 1972a). Mungal (1979) found that the minimum growth temperature of *Y. enterocolitica* was 1.4°C in TSB, but it increased to 9°C in TSB+5% NaCl and to 13°C in TSB+10% NaCl.

Many microbial activities are known to be affected by low temperatures. Increased production of the red pigment, prodigiosin, by *Serratia marcescens* at temperatures below the optimum for growth is due to the activity of an abnormally heat-sensitive enzyme (Rose, 1968). The optimum

temperature for production of flagella by *E. coli* is lower than the optimum growth temperature (Morrison and McCapra, 1961). In *Klebsiella pneumoniae*, an increase in the content of cell carbohydrate is observed when the temperature of a continuously growing culture is decreased from 35 to 25°C (Tempest and Hunter, 1965). Similarly, the production of extracellular dextrans by leuconostocs and pediococci is favored at lower temperatures.

The carbohydrate to protein ratio of *E. coli* cells growing at 10°C is 0.37 w/w, whereas at 37°C the ratio is 0.13 (Ng, 1969). While the growth rate of *E. coli* at 10°C is slower than that predicted by the Arrhenius equation (deviation begins at temperatures below 20°C), the respiratory rate seems to obey the equation to 10°C (Ng, 1969). The author suggests that the inability of biosynthesis to keep pace with catabolism results in energy being diverted into storage forms (e.g. glycogen), resulting in the higher carbohydrate to protein ratio at low temperatures.

2.1.3 Lipids and Solute Transport

Solute uptake by microorganisms can be affected by low temperatures (Farrell and Rose, 1967; Wilson *et al.*, 1970). The defect has been attributed to changes in the molecular structure of the cytoplasmic membrane, inhibiting carrier proteins of transport systems, which in turn may determine the minimum growth temperature (Farrell and Rose, 1967;

Wilson *et al.*, 1970). The fatty acid composition of bacterial membranes may be important in the determination of minimum growth temperature (Farrell and Rose, 1967; Rose, 1968). The composition of bacterial lipids varies markedly with conditions of growth (Davis *et al.*, 1980). At lower temperatures, microorganisms synthesize a larger proportion of unsaturated fatty acids which have a lower melting point. At higher temperatures the reverse is true (Davis *et al.*, 1980). This phenomenon has long been interpreted as a homeostatic mechanism.

A unique feature of phospholipid bilayers of the bacterial cell membrane is their ability to undergo a reversible thermotrophic gel to liquid-crystalline transition which arises from the cooperative melting of the hydrocarbon chains in the interior of the bilayer (Esser and Souza, 1976; McElhaney, 1976). The selective melting of the phospholipid fatty acid chains does not result in a gross molecular rearrangement, so that the general bimolecular leaflet structure exists both above and below the phase transition temperature (McElhaney, 1976). The temperatures at which phase changes occur depend upon the head group, the length, the degree of unsaturation of the hydrocarbon chain and the amount of branching (Esser and Souza, 1976). At lower growth temperatures, lower melting points and transition temperatures occur as a result of more unsaturated and branched fatty acids causing increased membrane fluidity (Esser and Souza, 1976; Kogut, 1980)

It is possible that the minimum growth temperature of an organism can be established by the lower boundary of the gel to liquid-crystalline phase transition. If set by membrane fluidity, the lower temperature limit for growth of bacteria might be governed by an organism's repertoire of membrane composition including phospholipid, intrinsic membrane proteins and cations (Kogut, 1980). *Corynebacterium xerosis* respires exogenous glucose and accumulates glucosamine at temperatures as low as 10°C, which is well below the minimum growth temperature for this mesophilic bacterium (Rose and Evison, 1965). Therefore the minimum growth temperature does not appear to be set by inhibition of membrane transport.

A shift in temperature of *E. coli* ML30 from 37°C to 10°C resulted in an alteration of the fatty acid composition of the cytoplasmic membrane during the 4.5 h lag period before growth recommenced at the lower temperature (Shaw and Ingraham, 1965). The fatty acids become more highly unsaturated during the first hour of this lag. Starvation of cells at 37°C, followed by replenishment of glucose at 10°C at the end of the lag period, allowed a dissociation of the resumption of growth from the change in fatty acid composition (Shaw and Ingraham, 1965). From this it was concluded that *E. coli* can grow at 10°C with the same fatty acid composition of the cytoplasmic membrane that it has at 37°C. The authors concluded that the minimum growth temperature is not determined by the fatty acid composition

of the membrane.

The membrane fluidity theory might define the range of permissive growth temperatures, but the minimum growth temperature is probably set at some temperature higher than that imposed by the solidification of the membrane (Esser and Souza, 1976; Inniss and Ingraham, 1978; Kogut, 1980). Temperature dependent changes in nucleic acids and proteins could also determine the minimum growth temperature (Esser and Souza, 1976).

2.1.4 Protein Synthesis and Activity

The synthesis and activity of enzymes in microbial cells are controlled by a complex array of regulatory processes, ensuring that the cell synthesizes just sufficient intermediates and end products to satisfy its metabolic requirements (Rose, 1968). Some of these regulatory processes are sensitive to temperatures below those optimum for cell growth. Ng *et al.* (1962) proposed that a major reason for the existence of a minimum growth temperature of 8°C in *E. coli* is that the regulation of enzyme synthesis becomes "deranged" at low temperatures. The induction of β-galactosidase is insensitive to glucose repression at temperatures below 20°C, the same temperature at which the rate of growth begins to deviate from that predicted by the Arrhenius equation.

E. coli was unable to grow at 13° with tryptophan as the sole carbon source because tryptophanase could not be

induced (Ng and Gartner, 1963). Mutants constitutive for tryptophanase were selected and were able to grow at 13°C. A failure of the operator gene to function at low temperatures was suggested as the reason for the defect (Ng and Gartner, 1963). Many proteins function at low temperatures but proteins which have evolved for optimum function at higher temperatures can, in certain instances, lose their function at low temperature (Ingraham, 1969).

Mutants with increased minimum growth temperature, called cold sensitive mutants, have proven useful in the study of the biochemical basis of the minimum growth temperature (Ingraham *et al.*, 1983). In most cases, the only detectable differences between mutant and parent strains are seen at the lower range of growth temperatures. Cold sensitive histidine mutants of *E. coli* and arginine mutants of *S. typhimurium* which require exogenous histidine and arginine for growth at low temperatures, respectively, have been isolated (O'Donovan and Ingraham, 1965; O'Donovan *et al.*, 1965; Abd-El-Al and Ingraham, 1969; Ingraham, 1969). In both cases, feed-back inhibition control was affected by low temperatures resulting in the cold sensitive phenotype. In these organisms, the minimum growth temperature was established by the low temperature sensitivity of particular enzymes.

Assembly processes have also been suggested to determine the minimum growth temperature. At 0°C, leucine-starved cells of an *E. coli* auxotroph incorporate

radioactive leucine into a wide variety of proteins (Goldstein *et al.*, 1964). The rate of protein synthesis at 0°C declines gradually until it comes to a complete halt after a few hours (Goldstein *et al.*, 1964; Das and Goldstein, 1968). Das and Goldstein (1968) suggested that ribosomes synthesize proteins at 0°C only until they run off a certain length of mRNA. At this point the ribosomes are free to be subjected to two opposing reactions: (1) inactivation and (2) reactivation. At 37°C the equilibrium is towards reactivation, at 0°C towards inactivation. When an exponential culture of *E. coli* was stored at 10, 12, 20 or 37°C for four hours, about 30% of ribosomes existed as 30S and 50S subunits as opposed to 70S ribosomal particles. But after 2.5 h at 8°C more than 80% of the ribosomes are present as subunits (Friedman *et al.*, 1969). Some step in the condensation of ribosomal subunits onto *E. coli* mRNA is prevented at low temperatures (Friedman *et al.*, 1969). Polysomes of *Azotobacter vinelandii* (Oppenheim *et al.*, 1968) and *Bacillus stearothermophilus* (Algranati *et al.*, 1969) disappear during cold storage.

Cold sensitivity can be caused by mutations in genes encoding ribosomal proteins (Guthrie *et al.*, 1969; Tai *et al.*, 1969). These mutants are unable to assemble ribosomal subunits at low temperatures. Instead, they accumulate incomplete, non-functional ribosomal subunits within the cell (Inniss and Ingraham, 1978). This suggests that the minimum growth temperature may be determined by the cold

sensitivity of polysome formation. That is, ribosomal subunits may not come together to make functional 70S ribosomes possibly due to an altered configuration of the ribosomal subunits at low temperature.

P. aeruginosa has detectable protein synthesizing activity even at 0°C (Saruyama *et al.*, 1979). Although protein synthesis by *P. aeruginosa* was completely lost after 12 h of chilling, the relative amounts of polysomes, monosomes and subunits did not change appreciably (Saruyama *et al.*, 1980). This suggests that the minimum growth temperature may be determined by different temperature-sensitive reactions in different organisms.

2.1.5 Morphology

The formation of filaments at temperatures below the minimum for growth has been observed in *E. coli* ML30 (Shaw, 1968). When an exponentially growing culture at 37°C is transferred to 6°C, DNA synthesis continues for 5 to 6 days while RNA and protein synthesis continues for about 8 to 10 days. If an exponentially growing culture at 18°C is transferred to 6°C, the synthesis of mucopeptide, RNA and protein continues for 13-14 days and DNA synthesis ceases after 10 days (Shaw, 1968). In both cases the cells become filamentous.

S. typhimurium experiences defective cell division during growth at 10°C (Hsu, 1972). After a lag of about 24 h, 75% of the cells undergo one division, after which

they elongate without dividing. This morphological change is temperature dependent and it is enhanced by nutrient limitation (Hsu, 1972). The filamentous growth resulting from low temperature incubation is reversible. A culture of these filaments reverts to rods after 4 hours incubation at 37°C (Hsu, 1972).

Mutants of *E. coli* altered in one or more aspects of cell division have been reported (Hirota *et al.*, 1968; Utsumi *et al.*, 1983; Shoemaker *et al.*, 1984; Tormo *et al.*, 1985). Nearly all of these mutants are conditional temperature sensitive mutants, i.e. they are unable to function at higher temperatures which results in their inability to divide (Hirota *et al.*, 1968). The major phenotypic characteristic of cell division mutants in *E. coli* is the production of long, non-septate, filamentous cells (Shoemaker *et al.*, 1984). In *E. coli*, the phenomenon of cell filamentation is classified into two types: one that occurs without inhibition of DNA replication, and the other that occurs with inhibition of DNA replication which results in the stoppage of septation, leading to cell filamentation (Utsumi *et al.*, 1983).

Low-temperature-induced filaments of *S. typhimurium* were able to revert to the rod forms in the presence of nalidixic acid, i.e. in the absence of DNA synthesis (Hsu, 1972). However, cell wall and protein synthesis are necessary for the filaments induced at cold temperature to revert back to rods upon incubation at 37°C (Hsu, 1972).

Ferroni and Inniss (1974) observed filament formation in *Bacillus insolitus*, a psychrophile, when it is incubated at 30°C. This filament formation can be reversed by incubation at 20°C, but not when *de novo* protein synthesis is prevented by chloramphenicol or when D-cycloserine, vancomycin or penicillin are present, suggesting that protein and mucopeptide synthesis are necessary for cross-wall formation and cell division (Ferroni and Inniss, 1974).

Peptidoglycan synthesis is a coordinated process involving the action of specific autolytic enzymes to produce new growing points and to initiate cross-wall formation (Weidel and Pelzer, 1964). Any alteration in the balance between autolysis and biosynthesis can result in the production of abnormal cell walls or lysis of the cells (Gilbert, 1984). Although the precursors needed for elongation and septation are known to be chemically similar, the enzymes required for their assembly are likely to be different (Vicente, 1984). Sublytic concentrations of many agents: e.g. furalocillin and piperacillin (Botta and Park, 1981) can interfere with cross-wall formation without affecting cell elongation (Gilbert, 1984). The inhibition of specific enzymes such as penicillin-binding protein 3 (PBP3) can result in filament formation (Spratt, 1977).

In *E. coli*, the products of more than 40 genes have been implicated in growth and division (Vicente, 1984). Some of these genes have been shown to be clustered on the *E. coli* genetic map, for example septum proteins (such as PBP3

and the *ftsA* gene product), peptidoglycan enzymes (*mur* gene product), membrane proteins (*surA* gene product) and SOS repair enzymes (*sulB* gene product) are all found at minute 2.5 (Vicente, 1984) and they are possibly under a common control mechanism.

2.2 Injury

The death of microorganisms due to many physical and chemical treatments is a gradual process and, if the process has not progressed too far, it can be reversed under appropriate conditions (Ray, 1979). Therefore, a population of bacterial cells that has been exposed to environmental stress may contain damaged cells and unharmed cells in addition to those killed by the treatment. Injury is observed when cells survive the stress but lose some characteristic ability to grow normally under conditions that are satisfactory for untreated cells (Busta, 1976, 1978). The expression of injury takes many forms, but in essence it is the loss of ability of living cells to form visible colonies under specified conditions (Hurst, 1977).

2.2.1 Causes of Injury

Hurst (1977) reviewed the causes of injury and stated that treatments which cause injury include heat, cold, drying, freeze-drying, freezing, cold shock, osmotic pressure and irradiation. In addition, adverse chemical treatments similarly kill and injure microorganisms, for

example, acidification/fermentation, food preservatives and disinfectants (Hurst, 1977). Because of the importance of refrigerated storage in maintaining microbial quality of foods, effects of low temperature storage on cell injury will be stressed.

2.2.2 Symptoms of Injury

One of the primary sequelae of injury is the inability of the injured cell to grow on selective media which support the growth of normal, unstressed or uninjured cells (Palumbo, 1986). Selective media are formulated to supress or prevent growth of one group of organisms while permitting the growth of another group (Difco Laboratories, 1984). One principle used in the selection of a particular type of organism from a mixed population is to use the inhibitory property of a specific chemical without which the medium would be suitable for many species in the sample (Difco Laboratories, 1984). This principle has been exploited in the development of selective media for the detection of *Salmonella*. A number of different compounds can be used to inhibit the growth of undesired bacterial species, including:

1. dyes (e.g. brilliant green),
2. heavy metals (e.g. bismuth),
3. chemicals (e.g. desoxycholate),
4. antimicrobial agents (e.g. chloramphenicol, lysozyme) (Koneman *et al.*, 1979).

Injured microorganisms become susceptible to a wide variety of selective media because of increased sensitivities to: salt and toxic chemicals such as NaCl, bismuth and selenite (Busta and Jezeski, 1963; Iandolo and Ordal, 1966; Roth *et al.*, 1973; Mackey and Derrick, 1982; Mossel and van Netten, 1984), surface active compounds such as bile salts, desoxycholate and lauryl sulfate (Clark and Ordal, 1969; Scheusner *et al.*, 1971; Stiles *et al.*, 1973; Zaske *et al.*, 1980a), antibiotics and antimicrobial agents (Zaske *et al.*, 1980a; Mossel and van Netten, 1984; Ray and Johnson, 1984) and increased sensitivity to dyes such as brilliant green and crystal violet (Scheusner *et al.*, 1971; Hurst, 1977; Mossel and van Netten, 1984). Thus, a medium that may be quite suitable for undamaged cells may become unsuitable for injured cells (Hurst, 1977). This inability may be measured by a lack of growth on solid media or in broth media (Busta, 1976).

Injury can be monitored by a dual plating technique. Plating the sublethally stressed culture on a non-selective medium allows the total viable cells to be enumerated, and simultaneously on a selective agar allows those microorganisms in the culture which can tolerate the selective agent to be enumerated. The difference between these two plate counts represents the injured cells that are unable to initiate growth in the presence of the selective agent (Witter and Ordal, 1977). Sublethally impaired microorganisms repair the injury under appropriate

conditions, but prior to such repair they will not grow on selective media (Russell, 1984). This type of injury has been termed "structural" injury because it is supposed to be due to damage to the cells which allows the selective agent to act on the cells (Ray and Speck, 1973; Beuchat, 1978; Zaske *et al.*, 1980b). Damage to surface structures (lipopolysaccharides in gram-negative and teichoic acids in gram-positive bacteria) and the cytoplasmic membrane probably play the most important role in the failure to detect injured cells by many of the selective media (Ray, 1979; Ray, 1986).

Injured cells are no longer able to exclude the selective chemicals from entering the cells and the normally resistant cells become sensitive (Ray, 1979; Zaske *et al.*, 1980b). Because the injured cells cannot repair in the selective environment, they fail to multiply and will remain undetected on the selective media (Maxcy, 1970; Ray and Speck, 1973; Klein and Wu, 1974; Ray, 1979).

Injury may be manifested by an inability of the microorganisms to form colonies on a defined medium while retaining the ability to do so when complex nutrients are supplied (Busta, 1978). The recovery of freeze-injured *E. coli* was better on complex than minimal media (Straka and Stokes, 1959). The addition of enzyme digests of casein to minimal media led to improved recovery of injured organisms (Straka and Stokes, 1959; Ray and Speck, 1973) suggesting that the injured cells had a nutritional requirement for

peptides, i.e. they had lost their ability to use inorganic nitrogen. Ray and Speck (1973) defined these organisms as "metabolically injured".

Inhibition of the recovery of injured microorganisms on complex media has also been shown (Alper and Gillies, 1958; Tang and Jackson, 1979a). This response is called "minimal medium recovery". Typically, treatments which result in impaired synthetic ability (e.g., UV irradiation) demonstrate this effect (Harris, 1963). The injured cells when placed in a nutrient rich medium grow, but many of the required enzymes and intermediates are present in minimal concentrations (Harris, 1963) leading to a "suicidal" growth response.

Following injury, microorganisms are often more sensitive to additional stresses that are imposed upon them. Ray and Speck (1973) found that freeze-injured *E. coli* give much higher plate counts on violet red bile and desoxycholate lactose agars when the samples are surface plated rather than pour plated. This increased sensitivity is attributed to exposure to the hot medium used in pour plating technique. Freeze-injured cells of *Salmonella anatum* are highly sensitive to lysozyme, whereas unfrozen, fresh cells are not (Ray *et al.*, 1972). In *E. coli*, the extent of growth inhibition by fatty acid antimicrobial food additives (e.g. decanoic acid) is increased if the cells are chilled prior to exposure to the antimicrobial agent (Fay and Farias, 1976). Storage at 3°C allowed a culture of

Streptococcus lactis to maintain its viability (Speck and Cowman, 1970). However, the culture's ability to produce acid on subculture in milk decreased due to inactivation of a membrane proteinase as a result of the low temperature storage (Speck and Cowman, 1970).

Damaged cells may exhibit an extended lag phase following transfer to non-restrictive conditions (Jackson, 1974; Busta, 1978). The extended lag phase is thought to represent the time required for repair of the damage before growth is possible. Bacterial cells are classified as injured rather than dead when they are damaged, because they have the capacity to function in an unrestricted environment and restore a normal physiological state concomitant with initiation of growth (Busta, 1976). By this definition, the damage disappears upon cell division, which supports the contention that it is an injury phenomenon and not mutation that is being observed (Busta, 1976). Restoration of the undamaged state has been called "resuscitation" (Allen *et al.*, 1952), to stress the fact that the injured cells are being revived from apparent death (Busta, 1976).

2.2.3 Cold Shock

When exponential phase cells of *E. coli* are suddenly chilled, the majority of the cells are subsequently unable to form colonies on nutrient agar (Meynell, 1958). Organisms in the stationary phase of growth are completely resistant (Meynell, 1958). This phenomenon, known as cold shock, is

usually observed in gram-negative bacteria, but it has also been observed in some gram-positive bacteria (Mackey, 1984). Meynell (1958) concluded that the lethal effect of cold shock may be caused by interference with the control mechanisms of bacterial permeability. The death of cold shocked cells is accompanied by losses of protein, nucleic acid and polysaccharides from the cells (Ingram and Mackey, 1976). Strange and Postgate (1964) demonstrated that ribonuclease could penetrate cold shocked cells of *Klebsiella pneumoniae*. They also showed that endocellular protein became accessible to the dye anilino-napthalene-sulfonate. Thus, movement in and out of cold shocked cells is affected.

Modifying fatty acid composition (for example, by alteration in growth temperature) allows otherwise sensitive bacteria to become resistant to cold shock. The critical factor appears to be rapid cooling through the temperature zone in which membrane lipids undergo a phase transition from liquid-crystalline to gel states (Mackey, 1984). Rapid cooling fixes the lipids and proteins of the membrane in a random disordered state resulting in membrane leakiness (Mackey, 1984). The loss of endocellular constituents during rapid chilling may be a cause rather than an effect of bacterial death. Leakage occurs from rapidly chilled bacteria in relatively dense suspensions which remain viable. In dilute suspensions the concentration of leakage products in the suspending fluid is too low for resorption

to occur and loss of viability results (Strange and Ness, 1963).

Rapid chilling of cells of *S. heidelberg* in the mid-log phase of growth from 37°C to 5°C in glucose salts broth results in cold shock (Tang and Jackson, 1979a). The chilled cells grow better on a simple medium (glucose-salts agar) than on a complex medium (tryptic soy agar supplemented with yeast extract). Over an 8 h period, the cold shocked cells gradually recover their ability to grow on the complex medium when incubated at 5°C (Tang and Jackson, 1979a). This recovery process can be inhibited by rifamycin, implicating RNA synthesis as essential for recovery of the injured cells (Tang and Jackson, 1979a). Sato and Takahashi (1969) found that the viability of cold shocked cells of *E. coli*, *P. fluorescens* and *Bacillus subtilis* increases markedly when magnesium and ATP are added to the recovery medium. In DNA repair mutants of *E. coli*, the magnesium and ATP stimulated increase in viability is not seen (Sato and Takahashi, 1969). Sato and Takahashi (1970) suggested that a loss of magnesium from the cold shocked cell causes inactivation of the magnesium dependent DNA ligase, thus interfering with DNA synthesis and repair.

Mackey and Derrick (1986) found that cold shocked cells of *S. typhimurium* displayed "minimal medium recovery". Plate counts on M9 minimal agar were much higher than on tryptone soya yeast extract agar, a complex nutrient agar. The levels of peroxide in the different media varied considerably,

being much greater in the complex medium. The authors concluded that the minimal medium recovery phenomenon is a manifestation of peroxide sensitivity.

2.2.4 Chill Injury

Cells which show no immediate effects of chilling (e.g. stationary phase cells or slowly-cooled exponential phase cells) may be killed and injured if they are held for prolonged periods below their minimum growth temperature (Mackey, 1984). Cold storage of *Staphylococcus aureus* at 5°C causes injury and death of the microorganism (Jackson, 1974). The extent of death is influenced by the pH of the suspending medium. Viability, as determined by plate counts on trypticase soy agar, decreases most dramatically at pH 4, less rapidly at pH 5 and only gradually at pH 6, 7 and 8 (Jackson, 1974). Also, *S. aureus* stored at 5°C progressively lose their ability to form colonies on mannitol salt agar compared with trypticase soy agar (Jackson, 1974). Thus, a portion of the viable population is injured as a result of the cold treatment. Injured cells recover their ability to grow on mannitol salt agar within 2 h of incubation at 37°C, yet the culture does not increase in numbers on trypticase soy agar until 4 h after the shift to temperatures that permit growth of the organism.

S. aureus incubated in tryptic soy broth at 1, 3, 5 and 7°C become injured (Patterson and Jackson, 1979b). Injury, measured by the difference in plate counts on mannitol salt

and tryptic soy agars increases at the lower temperatures of storage (Patterson and Jackson, 1979b). Cells of *S. heidelberg* in the mid-log phase of growth are injured following cold storage at temperatures between 0 and 5°C (Tang and Jackson, 1979b). Peptidoglycan and ATP synthesis is necessary for resuscitation to occur when the cold storage menstruum is either glucose-salts broth or distilled water. However, cells stored in TSB are able to recover from the injury in the presence of inhibitors of ATP, peptidoglycan, DNA and RNA synthesis (Tang and Jackson, 1979a).

Damage to the cytoplasmic membrane can be expressed by leakage of intracellular materials including RNA, DNA, protein, enzymes, amino acids, potassium ions and/or adenosine 5'-monophosphate (Beuchat, 1978). Tang (1975) observed leakage of ultraviolet absorbing material from chill damaged *S. heidelberg*. In addition, anilino-naphthalene-sulfonate (ANS) enters the injured cells, which are normally impermeable to the dye, demonstrating that the permeability of the cold damaged cells is affected (Tang, 1975). Resuscitated cells regain their impermeability to the dye. A culture that is injured by storage in glucose-salt medium at 2°C for 4 days and is allowed to resuscitate for 20 minutes at 37°C, becomes as impermeable to ANS as a culture grown at 37°C. A culture that is injured by storage at 2°C for 10 days took longer to regain membrane impermeability to ANS (Tang, 1975). Thus, the extent of

injury increases with the time of exposure to low temperature. Other observations that suggest the involvement of membrane damage include increased sensitivity to sodium chloride, sodium desoxycholate and sodium lauryl sulfate (Beuchat, 1978). Chill injured *S. heidelberg* becomes sensitive to bile salts and sodium desoxycholate (Tang, 1975). He suggested that some steric or chemical change of the lipopolysaccharide layer of the cells allows the surface-active agents to come into contact with the membrane.

Growth conditions that influence the physiological state and composition of the cell may affect the susceptibility of the microorganism to damage by subsequent exposure to one or more stresses (Busta, 1976). Klaenhammer and Kleeman (1981) found that rough variants of *Lactobacillus acidophilus* RL8K are sensitive to freeze-damage at -20°C, whereas smooth colonial variants are resistant. Microscopically, rough cells are observed as long gram-positive rods with small non-stainable blebs protruding from the cell wall. Calcium supplementation of MRS medium causes a morphological transition of *L. acidophilus* NCFM from filaments to rods (Wright and Klaenhammer, 1981). The rods are more resistant to freezing and storage at -28°C. In addition, *L. acidophilus* is injured during frozen storage as shown by its increased sensitivity to MRS agar supplemented with 0.15% bile (Wright and Klaenhammer, 1981). These results show that cell wall damage occurs in *L. acidophilus*

as a consequence of growth conditions, possibly a calcium deficiency. This affects the morphology of the cells, which in turn restricts their ability to withstand additional stresses.

In general, cells in the exponential growth phase are more susceptible to death or injury than cells in the stationary phase (Dyer and Maxcy, 1982). When a stationary culture of *E. coli* is stored at 1 and 4°C only a slight decrease in viability and no evidence of injury is observed after 30 days. An exponential phase culture under the same conditions shows a dramatic loss of viability and extensive injury within 48 h. A similar response has been observed for *S. aureus*. However cells of *Streptococcus faecalis* are not affected in either of their growth phases (Patterson and Jackson, 1979a). A possible explanation for the resistance of *S. faecalis* to injury is that KF streptococcus agar, the medium used for selective isolation of this organism, is not particularly inhibitory. To demonstrate injury in stressed cells by differential plate counts requires that the non-selective medium is in fact non-selective, and that the selective medium has adequate selective potential to allow differences to be observed.

Dyer and Maxcy (1982) simulated the conditions of improper cleaning of food processing equipment to examine the fate of bacteria on the surfaces of food processing equipment in a refrigerated environment. At refrigerator temperatures, exponential phase cells were more sensitive

than stationary phase cells in a food film on stainless steel. The test organism was grown in either plate count broth or a filter sterilized beef "serum". Aliquots were transferred to test strips of stainless steel and refrigerated at 5°C until they were dry. Less death and injury was observed in the beef serum than in the plate count broth.

Water or diluents are more stressful environments than broth or food (Mackey, 1984). Mitchell and Starzyk (1975) found that the time required to reduce viable counts of *E. coli* and *S. typhimurium* by 90% at 0-5°C in water is between 12 and 16 days. On fresh beef, *S. infantis* and *S. aureus* were shown to survive prolonged refrigerator storage under both aerobic and anaerobic (vacuum-packaged) conditions (Kennedy *et al.*, 1980). Levels of *S. infantis* decreased by 52% in aerobically packaged and by 55% in vacuum-packaged beef stored at 1.7°C for 28 days (Kennedy *et al.*, 1980). Woodburn and Strong (1960) found that the survival of *Salmonella* spp. in buffer was greater if food was added to the buffer. They concluded that the chemical composition and the pH of the food ingredient added to the buffer affects the survival of the microorganisms.

Foster and Mead (1976) found that the survival of salmonellae at 1°C was greater in minced chicken breast muscle than in leg muscle. The authors suggested that the pH of the muscle caused the effect; the pH of breast muscle was 5.8 and that of leg muscle macerate was 6.4. By reducing the

pH of the leg muscle macerate to 5.8, greater survival was observed (Foster and Mead, 1976).

Oliver (1981) observed a dramatic loss of viability when *Vibrio vulnificus* was chilled in oyster homogenate at 4°C. The viable count decreased from *ca.* 10^7 colony forming units (cfu) per gram to undetectable levels within 24 h. Inoculation onto whole oysters resulted in a decrease from *ca.* 10^7 cfu/g to *ca.* 10^3 cfu/g, while inoculation into a salts broth resulted in a decrease to *ca.* 10^4 cfu/g following 24 h at 0°C (Oliver, 1981). He suggested that the accelerated death rate was due to toxic factors that were released upon chilling of the oysters and that it was active only on chilled cells. In chilled cells, sublethal injury can occur without loss of viability. Mackey and Derrick (1982) observed that the mild cold shock of diluting *S. typhimurium* grown at 37°C into room temperature saline instantly sensitized the cells to selenite without any loss of viability.

Salmonellosis is no longer transmitted by systemically infected meats but by contaminated products derived from perfectly healthy slaughter animals (Mossel, 1984). The behavior of salmonellae on these products until they are consumed is important in the control of food-borne salmonellosis. Prolonged refrigeration in water or broth causes extensive sublethal injury but it is not clear whether similar injury occurs during short-term (e.g. 1-2 weeks) refrigeration of foods, such as meat (Mackey, 1984).

3. MATERIALS AND METHODS

3.1 Test Organism and Growth Medium

Salmonella typhimurium strain 13311 from the American Type Culture Collection (ATCC) was used as the test organism throughout the study. Stock cultures were maintained on tryptic soy agar (TSA, Difco Laboratories, Detroit, Michigan) and were subcultured monthly.

Trypticase soy broth (TSB; BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, Maryland) was used to subculture the organism for all of the experiments.

3.2 Growth Response at Low Temperatures

The minimum growth temperature of *S. typhimurium* ATCC 13311 was determined in a temperature gradient incubator (Temperature Gradient Incubator, Scientific Industries Inc., New York). A 0.15 mL aliquot of the test strain was inoculated into a culture tube containing 15 mL of TSB which had been allowed to equilibrate in the temperature gradient incubator overnight. The temperature gradient was determined by measuring the temperature of a duplicate, interspaced set of tubes which contained water. The temperature of the tubes was determined daily using a probe thermometer (Caspar Integrated Systems, Fort Bragg, California). The tubes were shaken at *ca.* 50 strokes/min and growth was determined by measurement of OD_{600} at 24 h intervals for 13 days using a Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester,

New York). Smears of bacteria from each tube were gram stained either at the end of the 13 day incubation period or at the time that the culture had reached maximum absorbance. The gram-stained preparations were viewed using a phase contrast microscope. Cell dimensions were measured using an eyepiece micrometer (Spencer, Buffalo, New York) standardized by using a stage micrometer (American Optical Company, Buffalo, New York) with 0.01 mm divisions. The lengths of 100 cells that had been systematically chosen were determined.

3.3 Effect of Low Temperature Storage

3.3.1 Preparation of Inocula

A 0.1 mL aliquot of a 1/10 dilution of an overnight culture of the test organism was used to inoculate two bottles each containing 100 mL of sterile TSB. The inoculations were made 12 h apart. After 18-h incubation at 35°C, the first culture was used as the "stationary phase inoculum" (OD_{600} = 2.0); and after 6 h incubation at 35°C, the second culture was used as the "log phase inoculum" (OD_{600} = 0.15). A 1/10 dilution of these cultures was made in fresh TSB for use in the experiments.

3.3.2 Storage In Broth Menstrua

3.3.2.1 Inoculation of Storage Menstrua

The broth storage menstrua used in these studies were trypticase soy broth 30 g/L (BBL dehydrated medium, TSB) and 10% TSB using 3 g of dehydrated medium per liter. Stationary and log phase cultures were inoculated separately into 150 mL of TSB and 10% TSB in 300 mL capacity side-arm erlenmeyer flasks (Belco Glass Inc., Vineland, New Jersey) to give a final cell concentration of approximately 10^7 colony forming units (cfu)/mL in each flask. The inoculated flasks were placed into "Aquatherm" shaking water baths (New Brunswick Scientific Co., Inc., Edison, New Jersey) set at 4, 7 or 10°C, and agitated at 100 rpm and stored for up to 30 days.

3.3.2.2 Injury of Cells During Storage

Samples of the inoculated broths were taken periodically over a one month period. Serial dilutions were made in 0.85% saline, and 0.1 mL aliquots of appropriate dilutions were surface plated onto a non-selective medium: trypic soy agar (TSA) and onto selective media: bismuth sulfite agar (BSA), brilliant green sulfa agar (BGS) and desoxycholate agar (DA). BSA plates were incubated at 35°C for 48 h; the others were incubated at 35°C for 24 h. All plating media were purchased from Difco Laboratories Ltd., Detroit, Michigan and were prepared as recommended by the manufacturer.

Injury and death were determined from differential colony counts on the non-selective and selective media. The various states of the bacterial cells resulting from cold storage were described as:

1. uninjured cells - cells that grow on the selective media;
2. total cells (injured and uninjured) - cells that grow on TSA; and
3. injured cells - the difference between counts on TSA and each selective medium.

The plates were poured in an Edgegard Laminar Flow Hood (Baker Co., Inc., Sanford, Maine) and allowed to dry for 20-30 min. They were stored, inverted in plastic bags at 4°C. To assure a standard level of selectivity throughout the injury experiments, the selective agars were always prepared 2 days in advance of being used for inoculation for enumeration of the cells.

3.3.2.3 Resuscitation of Injured Cells

Resuscitation of sublethally injured organisms was accomplished by placing the chilled cultures in a controlled environment incubator shaker (New Brunswick Scientific Co., Inc., Edison, New Jersey) at 37°C and 100 rpm. Samples were taken at 30 min intervals and 0.1 mL aliquots of serial dilutions of the culture were surface plated on to TSA, BGS, BSA and DA and incubated at 35°C. The OD_{600} of the culture was measured at each sampling time. In the first experiment, sampling was terminated 4.5 h after the transfer to the

permissive growth temperature since an increase in OD was observed, indicating cell growth. Subsequently, a standard 5 h resuscitation period was adopted to avoid growth of the cells during the experiment.

3.3.2.4 Morphological Variation of Cells During Chilled Storage

A 9 mL aliquot of the stored culture was transferred into a glass centrifuge tube. One mL of Kellenberger's fixative (1% w/v osmium tetroxide in Kellenberger's buffer, pH 6.1 (Glauert, 1965)) was added to the tube. The culture with added osmium reagent was centrifuged for 10 min at 4,000 rpm in an IEC HN-SII centrifuge (Damon/IEC Division, Needham Heights, Massachusetts) and left for 18-24 h to fix at room temperature. After fixation, the supernatant was removed with a pasteur pipette and discarded. The fixed sample was then washed twice with Kellenberger's buffer as follows: enough of the buffer was added to the tube to cover the pellet, left for 15 minutes and removed with a pasteur pipette. Next, the pellet was dehydrated by replacing the buffer with ethanol. Starting with 25% ethanol with a minimum exposure time of 15 minutes, the fixed cells were dehydrated in a graded series of ethanol solutions (25%, 50%, 75%, twice in 90% and twice in undiluted, anhydrous ethanol). The cells were stored in the undiluted ethanol. The ethanol was removed from the tube containing the fixed sample using a pasteur pipette. The fixed, dehydrated pellet was resuspended in a small amount of anhydrous ethanol,

applied to an SEM stub and allowed to air dry. The air-dried stubs were then sputter coated with gold in a Semprep2 Sputter coater (Nanotech, Manchester, England) and examined by scanning electron microscopy with either a Cambridge S250 or S100 scanning electron microscope (Cambridge Instrument Co., Ltd., Cambridge, England).

3.3.3 Studies with Inoculated Chicken Meat

3.3.3.1 Preparation of Inocula

Log phase and stationary phase cultures were prepared as described in section 3.3.1. The log phase inoculum was prepared by centrifuging 25 mL of a log phase culture and resuspending it in 2.5 mL of 0.85% saline. The stationary phase inoculum was prepared by centrifuging 10 mL of a stationary phase culture and resuspending in 10 mL of 0.85% saline.

3.3.3.2 Preparation of Chicken for Storage Trials

Fresh, not previously frozen, boneless chicken breasts were purchased from a local supermarket and were prepared for inoculation as follows: the skin was removed and the breast meat was cut into portions weighing approximately 10 g each. The samples were placed into a sterile 1 L beaker and the beaker was covered with aluminum foil. The chicken pieces were γ-irradiated in a Gammacell 220 (Atomic Energy of Canada Ltd., Ottawa, Ontario) for 20.5 h resulting in exposure of the sample to approximately 750 krad. The

irradiated chicken pieces were aseptically distributed into Whirl-pak bags (Nasco, Systems Plus, Waterloo, Ontario) and 0.1 mL of either a log or a stationary phase cell suspension was spread over the upper (un-cut) surface of the chicken piece to give *ca.* 10^7 cfu/g. Inoculated chicken and uninoculated controls were stored at 4, 7 and 10°C and were sampled at the start of the experiment and periodically over a 20-day storage period.

3.3.3.3 Viability and Injury

Each sample was stored in a separate Whirl-pak bag. At each sampling time, 90 mL of 0.85% saline was added to the Whirl-pak storage bag and the sample was homogenized in a Colworth stomacher (A. J. Seward, London, England) for 3 minutes. Serial dilutions of the homogenate were made and 0.1 mL aliquots were surface plated in duplicate onto TSA, BGS, BSA and DA. For the uninoculated control samples, 1 mL of the lowest dilution was also plated onto the agar media.

3.3.3.4 Morphological Variation

For scanning electron microscopy, two or three 1 cm^3 portions of the chicken were cut from the inoculated surface (or upper surface for uninoculated sample) and placed in a glass vial. Kellenberger's fixative was added to cover the sample and this was then left to fix for 18-24 h at room temperature. The fixed samples were washed and dehydrated as described in section 3.3.2.4. The samples were stored in anhydrous ethanol. Prior to examination by SEM, the samples

were placed into microporous sample cups and were further dehydrated in a critical point drier (Polaron Jumbo Critical Point Drier, Watford, England). The samples were then mounted onto SEM stubs, sputter coated with gold and examined with a Cambridge S250 scanning electron microscope.

4. RESULTS

4.1 Low Temperature Growth

The effect of temperature on the growth of *S. typhimurium* 13311 in trypticase soy broth (TSB) was examined by measuring absorbance at 600 nm. The best fit lines obtained at each temperature are shown in Figure 1. For the temperature range 4.7 to 11°C, there was no increase in absorbance at 4.7°C, but increase in absorbance occurred at all other temperatures. Up to 7.4°C, the organism did not achieve maximum absorbance during the 13-day experiment, however at 8.1°C and above, maximum absorbance was achieved within the time limit of the experiment. Gram stained preparations of the cells were examined using phase contrast microscopy, and cell lengths were measured using an eye-piece micrometer. Considerable variation in the length of 100 systematically selected cells was observed, as shown in Table 1. Increases in absorbance at temperatures of 6.7, 7.4 and 8.1°C were accompanied by substantial increases in the overall length of the cells.

4.2 Injury and Death in Broth

Both TSB and 10% TSB were capable of supporting the growth of *S. typhimurium* 13311. This was shown in a preliminary growth curve where *ca.* 10^5 cfu/ml of an overnight culture was inoculated into TSB and 10% TSB and

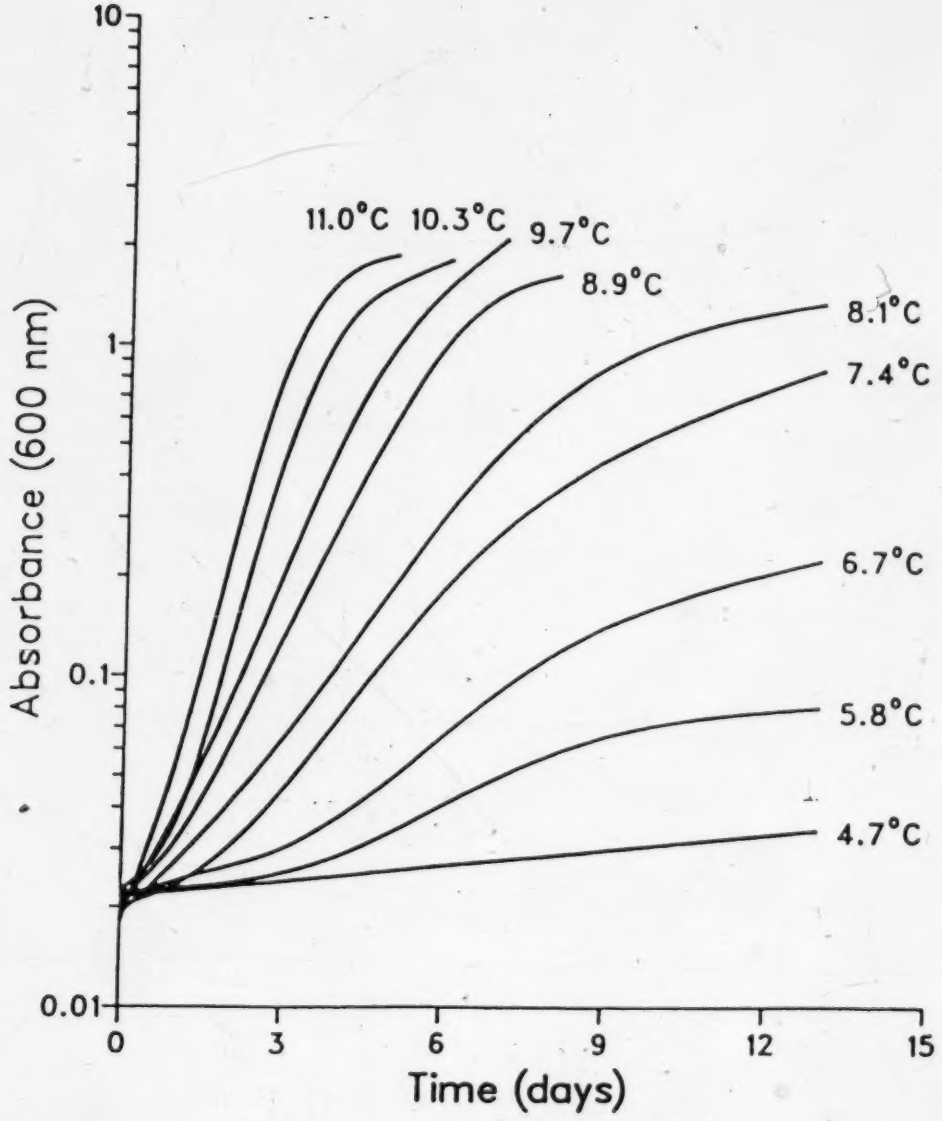


Figure 1. Growth of *S. typhimurium* 13311 in tryptic soy broth over the temperature range 4.7° to 11°C determined by absorbance (600 nm).

Table 1. The mean cell length of 100 systematically selected cells of *S. typhimurium* 13311 grown in TSB and incubated at different temperatures in a shaking (50 strokes/min) temperature gradient incubator.

Mean Temperature (°C)	Length of Cells (μm)	Range of Length Measurements (μm)
4.7 ± 0.4	2.55 ± 0.65	1.5 - 4.0
5.8 ± 0.4	3.50 ± 0.37	3.0 - 4.0
6.7 ± 0.4	11.83 ± 6.22	4.0 - 34.0
7.4 ± 0.4	18.10 ± 7.74	5.5 - 45.0
8.1 ± 0.4	10.34 ± 4.64	4.0 - 28.0
8.9 ± 0.5	4.93 ± 2.12	2.0 - 18.0
11.0 ± 0.5	2.33 ± 1.03	1.5 - 10.5
Inoculum	2.48 ± 0.55	1.5 - 4.0

grown at 35°C. The cell counts in the TSB culture reached $1.7x10^9$ cfu/ml while the 10% TSB culture grew to $1.8x10^8$ cfu/ml after 10.5 h incubation at 35°C. Thus, both broths were capable of supporting the growth of *S. typhimurium*. Incubation of these broths at 4 or 7°C, did not support growth of *S. typhimurium*, as measured by plate counts in fact both broths became stressful environments for the microbes.

4.2.1 Injury and Death at 4°C

The survival of log and stationary phase cells of *S. typhimurium* 13311 stored at 4°C in TSB and 10% TSB is shown in Figure 2. Stationary phase cells stored in either TSB or 10% TSB were resistant to the lethal effects of chilling. In contrast, log phase cells died during chilled storage. The lethal effect was greater in the more dilute (less cryoprotective) environment of 10% TSB. After 24 days at 4°C, there was 95% loss of viability in log phase cells stored in TSB and 98.5% loss of viability in 10% TSB, when compared with stationary phase cells stored in TSB at 4°C. The data presented in Figure 2 are representative of results obtained in two studies.

Injury of log phase cells of *S. typhimurium* 13311 during storage in TSB and 10% TSB at 4°C is demonstrated by differences in plate counts on non-selective (TSA) and selective (DA) media, as shown in Figure 3. Differences in plate counts on the non-selective and selective media are

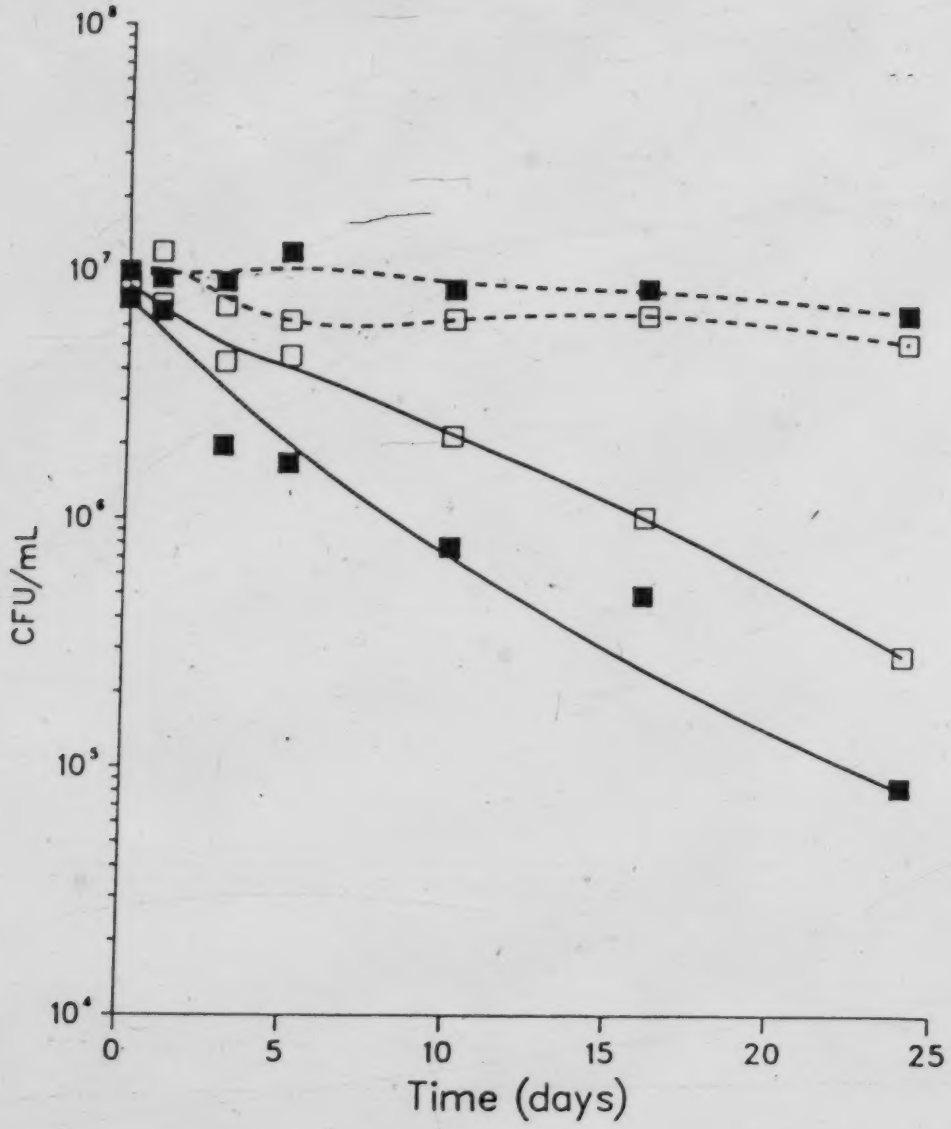


Figure 2. Viability of log phase cells (solid lines) and stationary phase cells (dashed lines) of *S. typhimurium* 13311 plated on TSA during storage at 4°C in TSB (□) or 10% TSB (■).

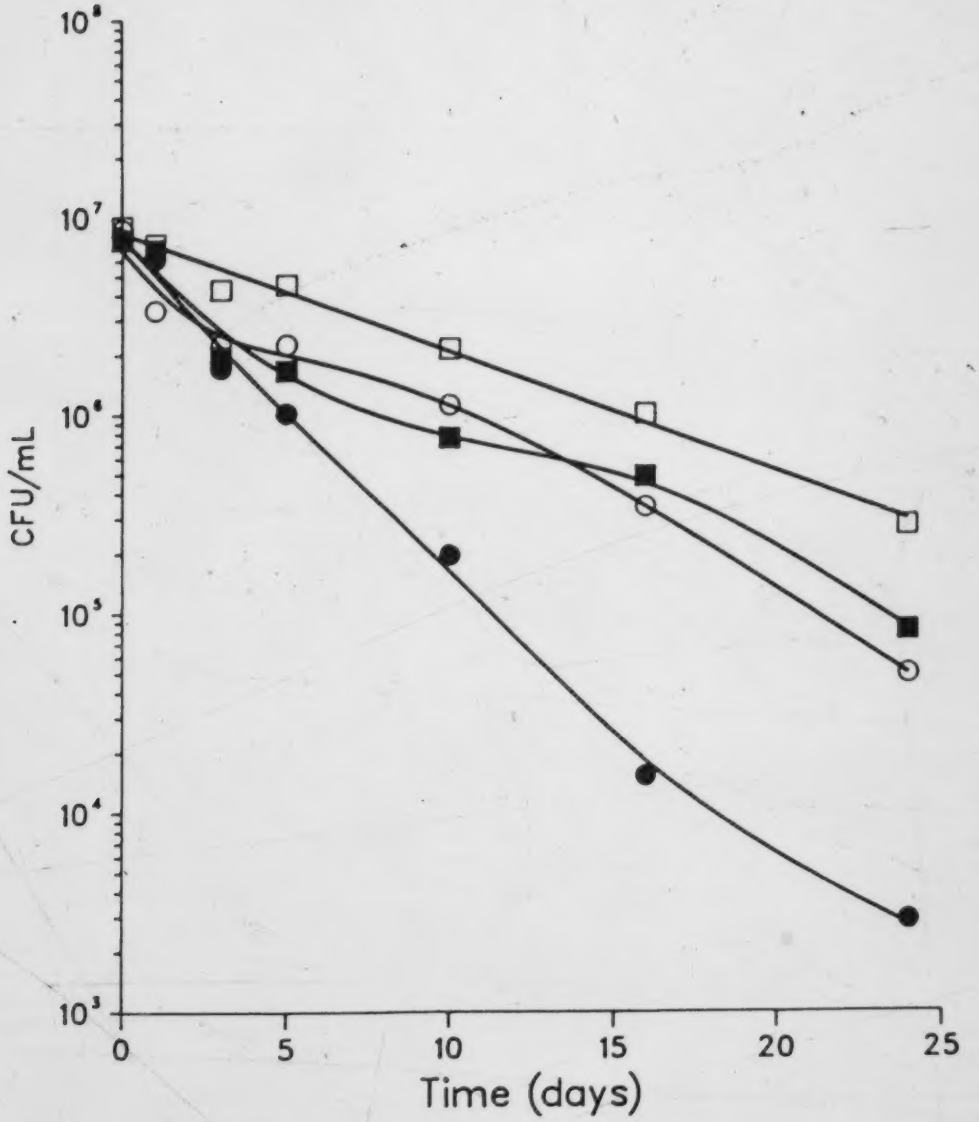


Figure 3. Death and injury of log phase cells of S. *typhimurium* 13311 during storage at 4°C for 24 days in TSB (open symbols) and 10% TSB (closed symbols) plated onto TSA (□, ■) and DA (○, ●).

negligible at the beginning of the chill treatment, but they increase with time of storage. After 24 days of storage the log phase cells in 10% TSB displayed marked injury (decreased growth on DA), and at the end of the experiment approximately 99% of the viable population was injured for growth on DA. The samples were also plated onto BGS and BSA, which resulted in 93% and 97% injury of the cells for growth on these media, respectively. The effects of chilled storage of log phase cells at 4°C in TSB are also shown in Figure 3. An 82% decrease in viability was observed on DA relative to counts on TSA. When the injured cells were plated on BGS and BSA selective media, the decreased viability due to cell injury was 59% and 82%, respectively.

The effect of chilling stationary phase cultures in TSB and 10% TSB is shown in Figure 4. Stationary phase cells stored in TSB for 24 days at 4°C did not exhibit decreased viability and injury was not detected on the selective media: DA, BGS or BSA. Stationary phase cells stored in 10% TSB did not show decreased viability initially, but after 15 days storage, injury of the cells occurred. At the termination of the experiment (24 days), 95% of the population was injured for growth on DA. Levels of injury on the other media were considerably less, only 24% on BGS and 80% on BSA. Two replicate studies gave similar results.

If the chilled cultures were subjected to conditions that would allow "resuscitation" such as incubating at 37°C in a shaking incubator, tolerance of the cells to the

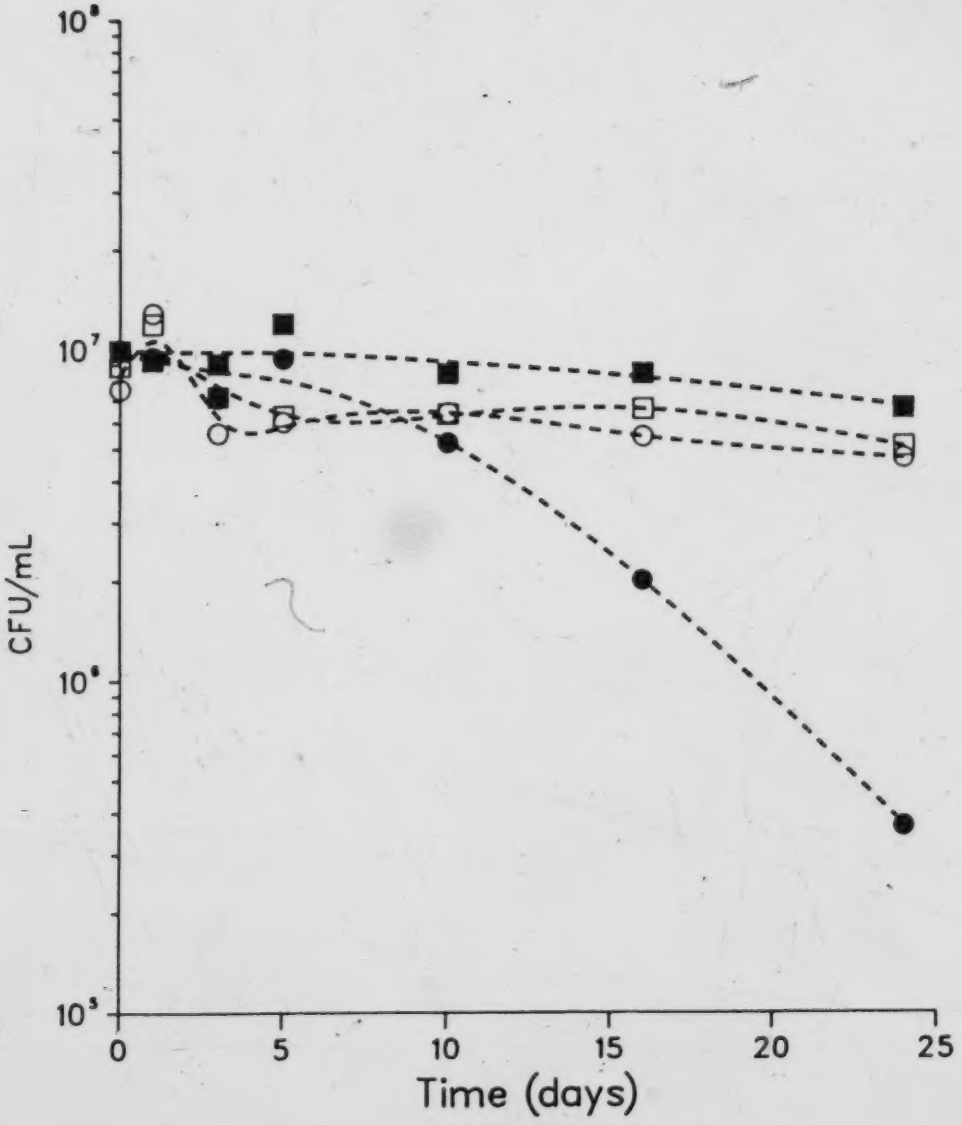


Figure 4. Death and injury of stationary phase cells of *S. typhimurum* 13311 during storage at 4°C for 24 days in TSB (open symbols) and 10% TSB (closed symbols) plated onto TSA (□, ■) and DA (○, ●).

selective media was restored (i.e. the cells resuscitated). Resuscitation curves for log phase cells injured by storage in TSB and 10% TSB are shown in Figure 5. The log phase cells that had been injured by storage in 10% TSB at 4°C recovered their ability to grow on DA after about 2 h incubation at 37°C. Growth of the culture did not occur, even after 5 h holding at the permissive growth temperature of 37°C, monitored by plate counts on TSA and by measurement of absorbance. Log phase cells in TSB were not injured as extensively as those stored in 10% TSB. After 35 d at 4°C, 86% of the cells were unable to grow on DA, and 78% were unable to grow on BSA. Cells injured during storage in TSB also regained their ability to form colonies on the selective agars in less than 2 h. However, growth of the cells occurred within about 2 h of the transfer to "resuscitation conditions". These data are representative of 3 separate resuscitation studies.

In the case of the stationary phase cells in 10% TSB, injured cells were able to form colonies on the selective agars after 2 h. Growth of the culture did not occur until 5 h after the change to "resuscitation conditions" (data not shown). Stationary phase cells in TSB were not injured for growth on the selective media and grew within 2.5 h in the resuscitation environment. Similar results were obtained in two separate trials.

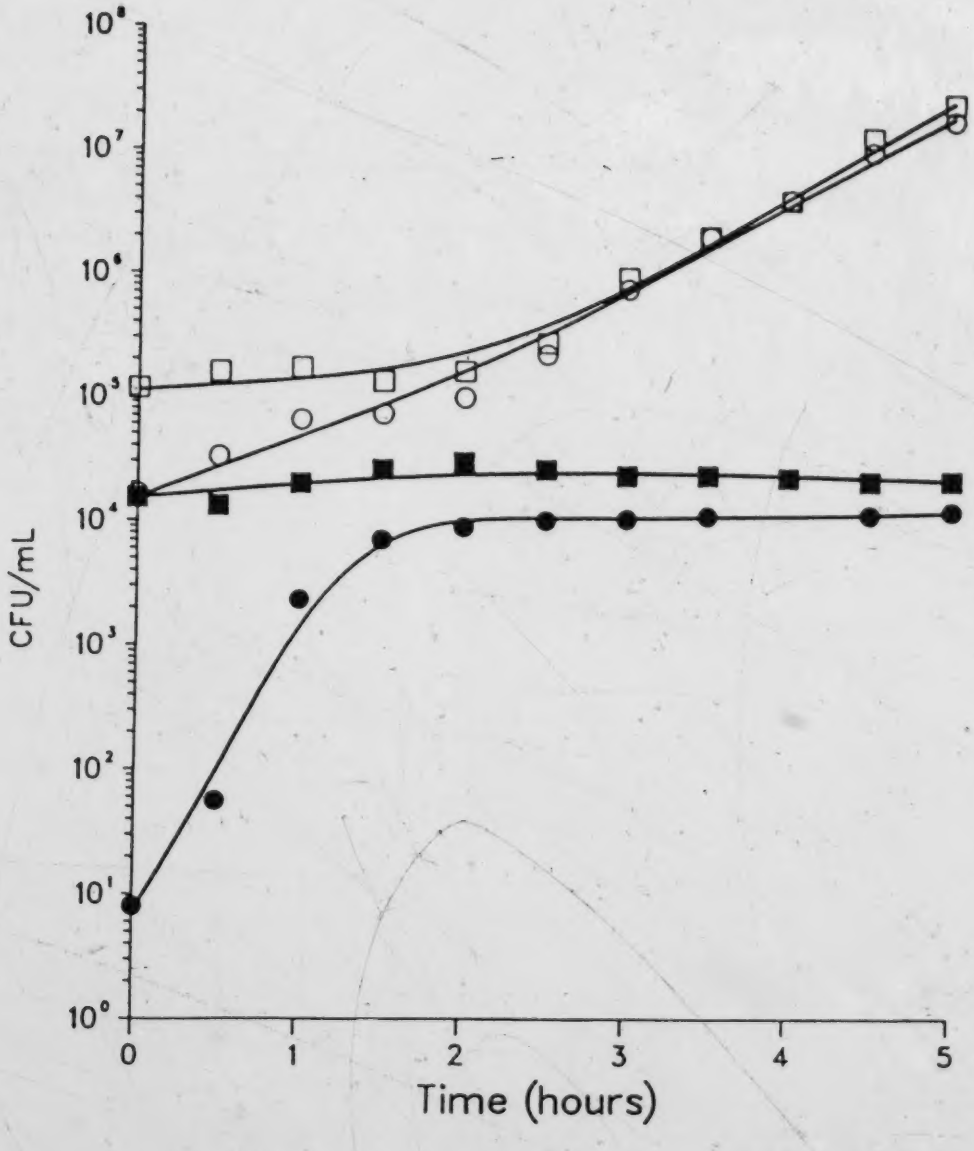


Figure 5. Resuscitation of log phase cells of *S. typhimurium* 13311 at 37°C, injured by storage in TSB (open symbols) and 10% TSB (closed symbols) at 4°C for 35 days. Resuscitation in TSB and plated onto TSA (□, ■) and DA (○, ●).

4.2.2 Injury and Death at 7°C

Storage of *S. typhimurium* 13311 in both TSB and 10% TSB at 7°C also resulted in loss of viability of cells on TSA, as shown in Figure 6. Cells stored in TSB were more resistant to injury due to the cold treatment than those stored in 10% TSB. In both storage menstrua, log phase cells were more susceptible to chilling than stationary phase cells. In comparison with stationary phase cells stored at 7°C for 25 days in TSB, log phase cells showed 75% loss of viability on TSA. Stationary and log phase cells stored in 10% TSB over the 26 days of the trial showed approximately 90 and 99% loss in viability, respectively.

In addition to the loss of viability at 7°C, *S. typhimurium* 13311 also became injured for growth on selective media, as illustrated by the data in Figure 7. The cells stored in TSB exhibited low levels of injury. Their counts on the selective agars were consistently lower than those on TSA (data not shown). Both log and stationary phase cells had plate counts that were lower on the selective agars, but even after 24 days of cold storage the differential between the selective and non-selective plate counts was less than 0.5 log cycle. After 24 days at 7°C, log phase cells had plate counts that were lower than counts on TSA by 23% on BGS, 35% on DA and 48% on BSA compared with counts on TSA. For stationary phase cells in TSB, 15% inhibition occurred on BGS, 20% on DA and 55% on BSA.

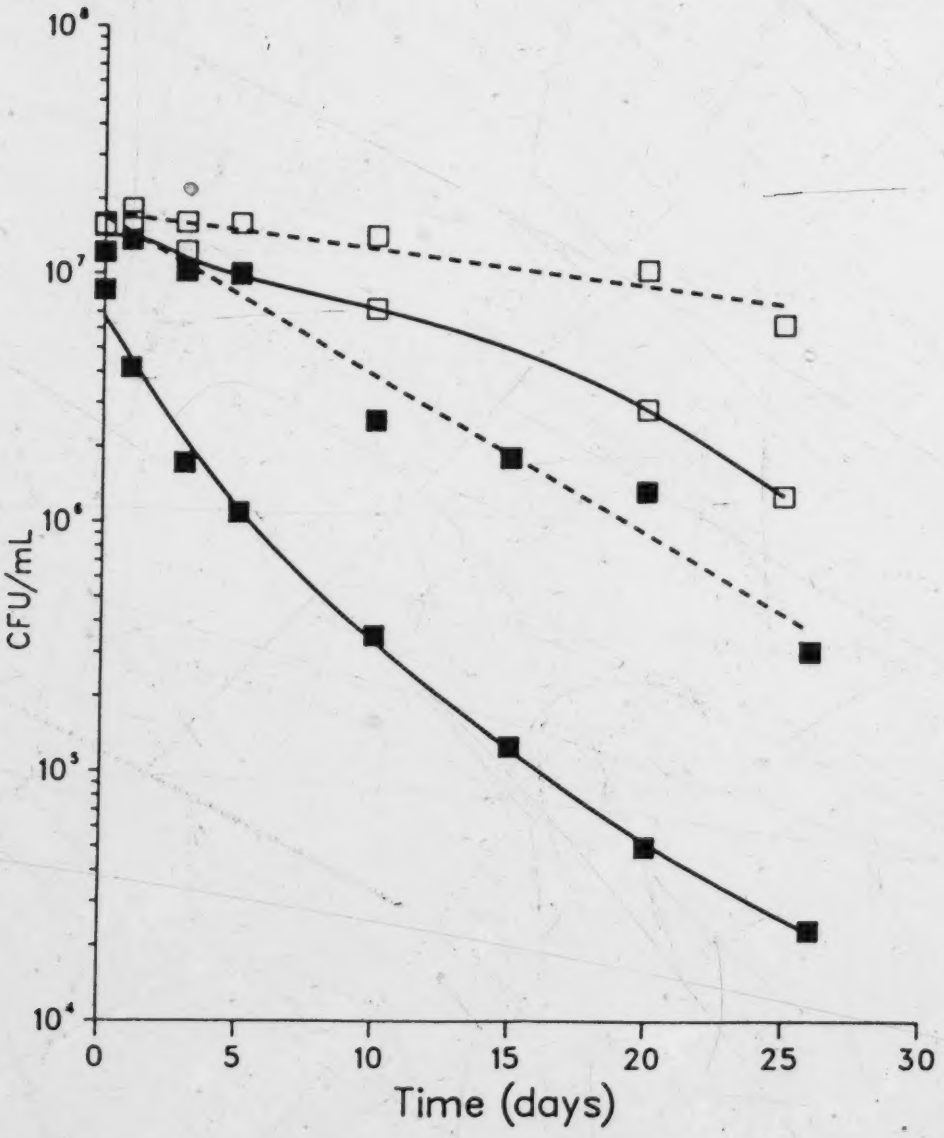


Figure 6. Loss of viability (ability to grow on TSA) of log phase cells (solid lines) and stationary phase cells (dashed lines) of *S. typhimurium* 13311 during storage at 7°C for 26 days in TSB (□) or storage at 7°C for 24 days in 10% TSB (■).

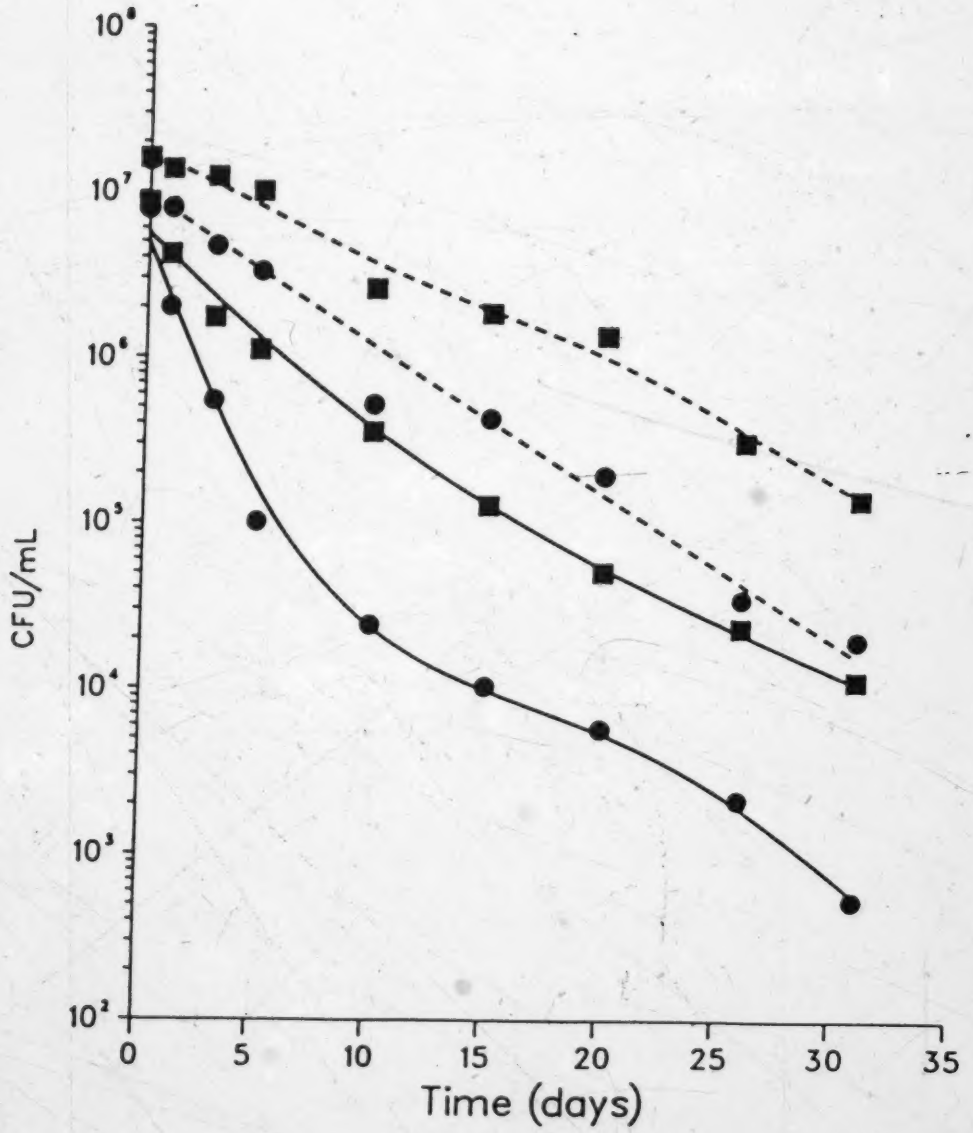


Figure 7. Death and injury of log phase cells (solid lines) and stationary phase cells (dashed lines) of *S. typhimurium* 13311 during storage at 7°C for 31 days plated onto TSA (■) and DA (●).

When *S. typhimurium* 13311 was stored in 10% TSB at 7°C, higher proportions of the surviving cells were injured compared with TSB as the storage menstruum. After 26 days at 7°C, the plate counts of stationary phase cells in 10% TSB were decreased by 69% on BSA and by 89% on DA compared with plate counts on TSA, as shown in Figure 7. Log phase cells in 10% TSB were the most sensitive to both killing and injury during 7°C storage. Of the surviving cells, 70% did not form colonies on BSA and 83% failed to form colonies on DA (Figure 7). The results presented in Figure 7 include injury data at 31 days of storage in addition to that obtained from the original viability/injury assay that was concluded after 26 days.

Log phase cells of *S. typhimurium* 13311 which had been injured by chilling at 7°C in 10% TSB could repair the damage when the chilled culture was held at 37°C in a shaking incubator at 100 rpm (Figure 8). Partial restoration of colony forming ability on BSA and DA occurred during a 3 h resuscitation period, at which point growth of the culture occurred. Resuscitation of stationary phase cells in 10% TSB was studied in the same way.

Brilliant green sulfa agar was not a good medium for measuring injury or resuscitation. In many cases, the injured cells displayed the phenomenon known as "communal growth response" (Roth *et al.*, 1972). Ten-fold dilutions of the chilled culture did not always give a ten-fold difference in counts on plating media. Results of this type

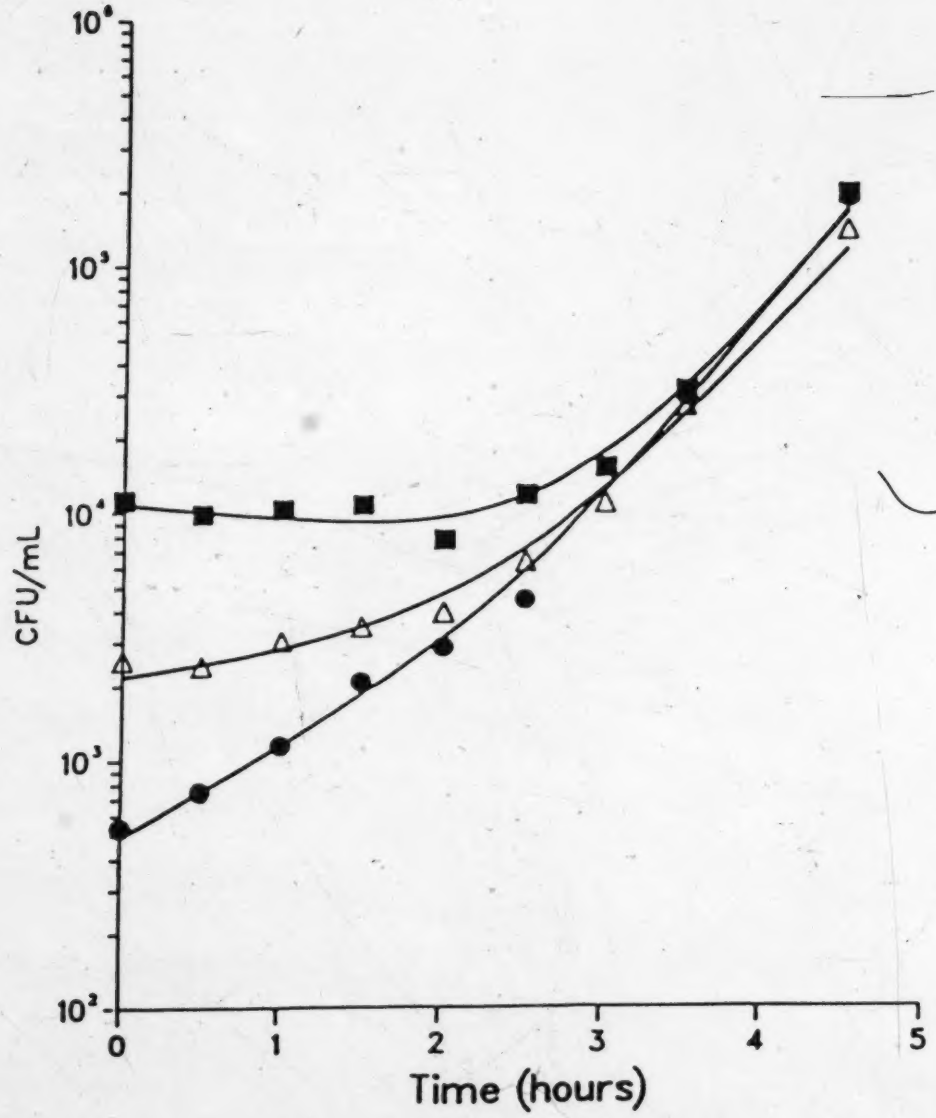


Figure 8. Resuscitation of log phase cells of *S. typhimurium* 13311 at 37°C, injured by storage in 10% TSB at 7°C for 31 days, resuscitated in 10% TSB and plated onto TSA (■), DA (●), BSA (Δ).

were frequently obtained during resuscitation experiments. This irregularity in the cell counts between dilutions cell injury. After resuscitation of the injured cells this type of growth response on selective agar was no longer seen.

4.3 Studies on Chicken Meat

Log or stationary phase inocula of *S. typhimurium* 13311 were applied to the upper (uncut) surface of a 10 g piece of chicken breast to give *ca.* 10^7 cfu/g. The inoculated chicken pieces were stored at 4, 7 or 10°C. Results of cold storage of *S. typhimurium* 13311 on chicken meat are shown in Figure 9. At 10°C there was growth to levels approaching 10^{10} cfu/g within 5 days. At 7°C growth occurred, but the cells did not achieve maximum population within 20 days. At 4°C there was no growth. Colony counts remained at *ca.* 10^7 cfu/g throughout the experiment. At the same time as viability was determined by plate counts on TSA, the homogenized samples were plated onto DA, BGS and BSA to see if the organisms had a decreased ability to form colonies on the selective media. Plate counts on the selective media were virtually the same as those on TSA. Data for log and stationary phase cells stored on chicken at 4°C, are shown in Table 2. It can be seen that the plate counts on DA are almost the same as those on TSA, not varying by more than 20%. This experiment was performed on two occasions with similar results.

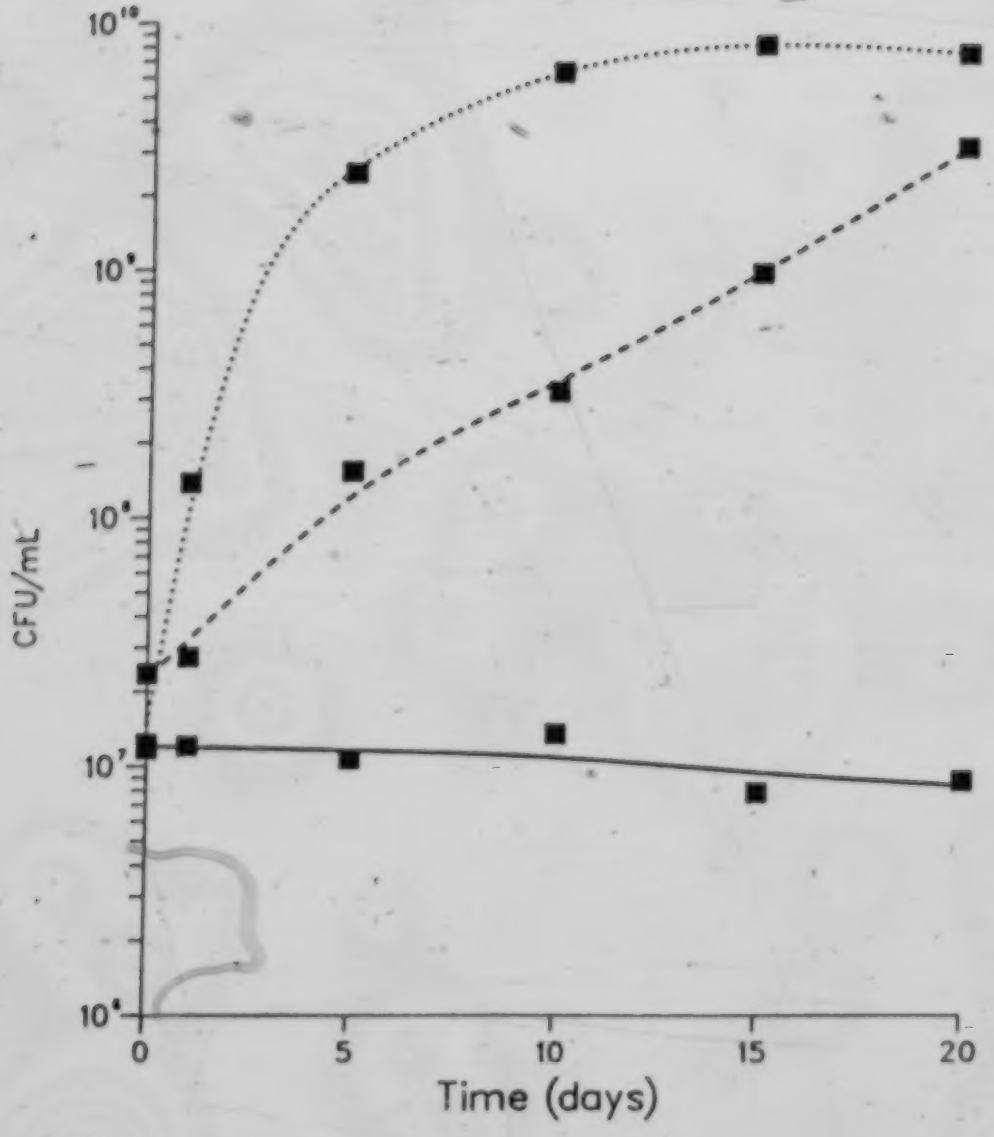


Figure 9. Viability of log phase cells of *S. typhimurium* 13311 inoculated onto the surface of irradiated chicken and stored at 4°C (solid line), 7°C (dashed line) and 10°C (dotted line) determined by plate counts on TSA.

Table 2. Effect of plating medium on counts of *S. typhimurium* 13311 inoculated onto the surface of irradiated chicken and stored at 4°C for 20 days.

Time of Storage (days)	Log phase cell (cfu/g)		Stationary phase cells (cfu/g)	
	Plating Medium			
	TSA	DA	TSA	DA
0	1.2×10^{7}	1.0×10^{7}	1.4×10^{7}	1.1×10^{7}
10	1.4×10^{7}	1.2×10^{7}	9.2×10^{6}	8.9×10^{6}
20	8.8×10^{6}	7.1×10^{6}	6.6×10^{6}	7.1×10^{6}

4.4 Morphology of Chilled Cells

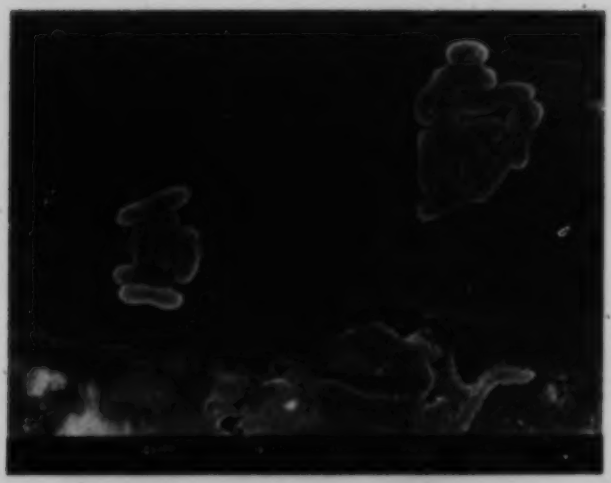
Scanning electron micrographs of broth cultures of *S. typhimurium* are presented in Plate 1. Dramatic differences in the morphology of the chilled cultures in broth were seen at the different chilling temperatures. When stored at 4°C in TSB, the cells remained as short rods approximately 2 μm in length (Plate 1, top). At 7°C, cell elongation was observed. Some of the cells developed into filaments up to 30 μm in length (Plate 1, middle). When incubated at 10°C, the cells consisted almost entirely of rods (Plate 1, bottom) of approximately the same length as those stored at 4°C and those used as the inoculum (Plate 2, top, left and top, right). Similar results were obtained for cells incubated at both 4 and 7°C, and for log phase cells in TSB and both log and stationary phase cells in 10% TSB. Only stationary phase cells in TSB were examined at 10°C. In plate 2, electron micrographs are shown of stationary phase cells that had been inoculated onto irradiated chicken meat and stored at 7°C for one day (middle, left) and 15 days (middle, right) or 10°C for one day (bottom, left) and 15 days (bottom, right). It can be seen that even after one day of storage at 7°C, there was already increased length compared with the length of the cells in the inoculum (top, left). On the top, right of Plate 2, the stationary phase inoculum is shown at approximately the same magnification as the cells stored on chicken for 15 days. After 15 days storage at 7°C, the cells became very elongated, many were

Plate 1. Scanning electron micrographs of stationary phase cells of *S. typhimurium* 13311 during storage in TSB.

Top : 4°C for 16 days

Center : 7°C for 21 days

Bottom : 10°C for 21 days



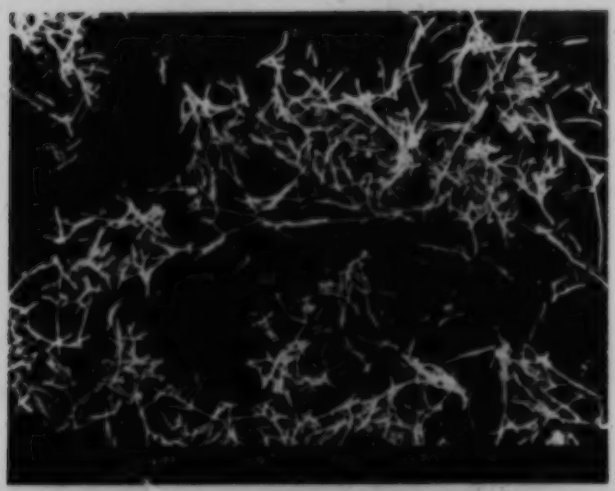


Plate 2. Scanning electron micrographs of stationary phase cells of *S. typhimurium* 13311 during storage on chicken meat under specified conditions.

Top left **Top right**

Stationary phase cells grown in TSB at 35°C for 18 h, and used to inoculate the meat pieces.

Center left **Center right**

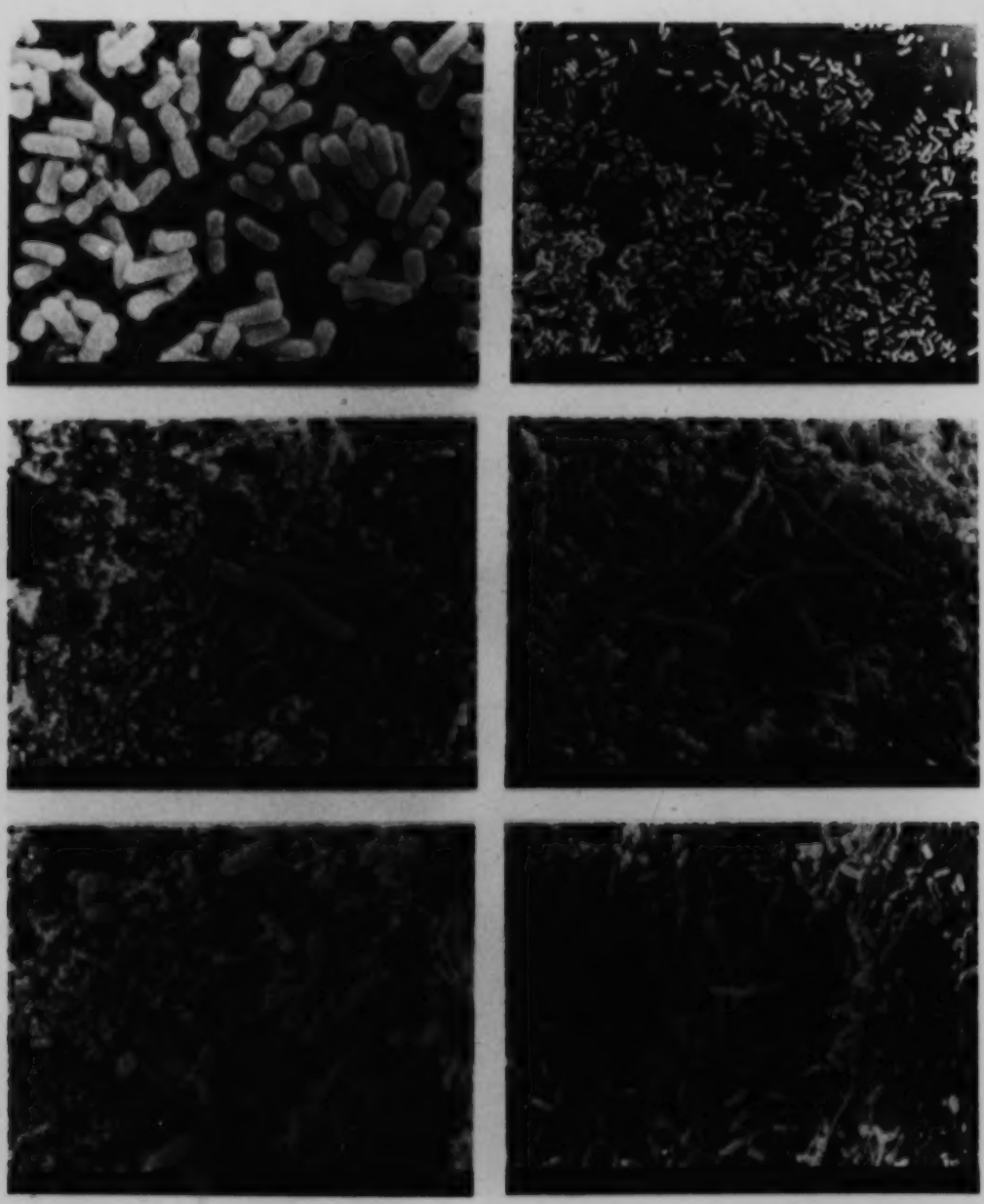
Cells on inoculated chicken pieces, stored at 7°C

1 day 15 days

Bottom left **Bottom right**

Cells on inoculated chicken pieces, stored at 10°C

1 day 15 days



greater than 20 μm.

Plate 2 (bottom, left) shows stationary phase cells following one day incubation on chicken at 10°C. After 15 days at 10°C, the cells were present mostly as short rods, with some elongation (Plate 2, bottom, right). For inoculated chicken stored at 7° and 10°C, increases in plate counts were observed during incubation (Figure 9) and the electron micrographs in Plate 2 suggest that changes in morphology of the cells likely accompanied those increased plate counts, particularly at 7°C.

5. DISCUSSION

The main purpose of the present work was to examine the effect of suboptimal temperatures on *Salmonella typhimurium* 13311. Three temperatures were selected for the study: 4°C, a temperature which represents adequate refrigeration; 7°C, an inadequate refrigeration temperature; and 10°C, an abusive storage temperature. In commercial practice, conditions of cold storage used for poultry meat are subject to fluctuation during transportation and retail storage (Foster and Mead, 1976). Greer (1984) demonstrated that the temperature of steaks in a retail storage case was generally about 9°C higher than that indicated on the "blower" thermometer on the retail display unit. For example, storage of meat at an acceptable blower temperature of 2°C resulted in a steak temperature of 11.8°C. Therefore, instead of inhibiting growth, retail storage of meats might provide marginal growth temperatures for salmonellae. Kraft (1971) showed that salmonellae were recovered more frequently from turkey meat from freshly-killed turkeys that had been chilled overnight than from frozen carcasses.

Since conditions of low temperature stress affect the mechanism controlling cell division in *Salmonella typhimurium* 13311 (Hsu, 1972) and in *E. coli* (Shaw, 1968), the morphology of the cells at lower temperatures was examined. *S. typhimurium* was incubated at low temperatures in TSB to establish the range of temperatures at which cell elongation would occur. At temperatures below *ca.* 4.7°C the

cells were the same length as those in the stationary phase inoculum. These cells might be described as being held below their minimum growth temperature. At temperatures higher than 4.7°C but lower than 11°C increases in absorbance were accompanied by elongation of the cells. Since plate counts were not performed, it is not possible to say whether the increases in absorbance were due to cell elongation or increased cell numbers. However, at temperatures above 11°C, the mean cell length was approximately the same as that of the stationary phase inoculum, suggesting that increases in OD_{600} at these temperatures was the result of increased cell numbers.

Both morphology and plate counts of chilled suspensions were observed in the viability/injury assays at 4, 7 and 10°C. The cells stored in broths at 4 and 10°C were approximately the same length as those in the inoculum. But, at 7°C, in both TSB and 10% TSB there were increases in cell length. However, there was a rapid decrease in the viability of the culture. Similarly, increased length with decreased viability of *E. coli* ML30 was observed following transfer of cultures to 6°C (Shaw, unpublished results, cited in Shaw, 1968). Shaw (1968) suggested that unbalanced growth of the culture was responsible for this observation. The formation of the filamentous cells appears to be a growth phenomenon. However, it does not appear to be lethal to the cells, as suggested by studies in broth (Shaw, unpublished results, cited in Shaw, 1968) and in this study. Filaments were also

observed on inoculated chicken stored at 7°C. However, these cells increased in numbers as well as in length.

Both TSB and 10% TSB were capable of supporting the growth of *S. typhimurium* 13311 at 35°C. However, neither TSB nor 10% TSB supported growth of *S. typhimurium* at 4 or 7°C, in fact both broths become stressful environments for the bacteria. Since TSB contains cryoprotective components (e.g. carbohydrates, peptides) it was assumed that by reducing the concentration of the ingredients in the broth to 10%, the amounts of the cryoprotective substances would be reduced, resulting in conditions that would be more stressful to the test organism at low temperatures.

Cryoprotectants are of two types: those capable of penetrating the cell (e.g. glycerol and dimethylsulfoxide) and non-penetrating compounds (e.g. polyvinylpyrrolidone (PVP) and dextran) (Ray, 1983; Mackey, 1984). Permeating cryoprotectants enter the cell and act by protecting the cells from the effects of osmotic stress by being concentrated both intracellularly and extracellularly (Mackey, 1984). The non-permeating cryoprotectants act by providing a physical barrier around the surface of the cell (Mazur, 1977). In these experiments, cryoprotective action might be attributed to both mechanisms. Limited solute uptake by microorganisms at temperatures below their minimum growth temperature has been observed (Rose and Evison, 1965; Shaw and Ingraham, 1967). Also, the broths were capable of supporting growth of the bacteria upon transfer to a

non-restrictive temperature. Thus, some of the nutrients were still present in the broth after storage for 30 days. It has been suggested that cryoprotectants of both kinds protect the LPS layer of the outer membrane of gram-negative bacteria and the teichoic acids of gram-positive bacteria from damage during cold treatments (Kempler and Ray, 1978; Ray, 1983)

In a preliminary experiment, stationary phase cells held in TSB at 4°C did not lose viability or show signs of injury following prolonged chilling at 4°C. Others studying cold temperature effects on *Salmonella* spp. were able to demonstrate injury at refrigeration temperatures (e.g. Tang, 1975). This author used log phase cultures of *S. heidelberg* to demonstrate injury in TSB and a glucose-salt broth. Many workers have found that bacterial cells in the exponential growth phase are more susceptible to injury than cells in the stationary growth phase (Sherman and Albus, 1923; Srivastava and Thompson, 1965; Hurst *et al.*, 1974; Patterson and Jackson, 1979a; Dyer and Maxcy, 1982). Extensive biosynthetic activity within growing cells as well as changes in the osmotic barrier during cell division are thought to make exponential phase cells more susceptible to injury than stationary phase cells (Hansen and Riemann, 1963). But the opposite has also been shown (Fay and Farias, 1976; Hagen *et al.*, 1976).

Log phase cultures of *S. typhimurium* were compared with stationary phase cells to see if physiological age had an

effect on loss of viability and expression of injury. In this study, log phase cells were more sensitive than stationary phase cells to chilling at 4° and 7°C in TSB and 10% TSB as shown by loss of viability on non-selective media and injury to growth on selective media. Thus, it appears that metabolically active exponential phase cells are more susceptible to chilling temperatures than the relatively inactive stationary phase cells. In many studies of the lethal effect of low temperatures, it has been shown that bacteria are most sensitive at temperatures just below their minimum temperature for growth (Mackey, 1984; Ingram and Mackey, 1976). But this is not always the case. Patterson and Jackson (1979b) found that survival of *S. aureus* in broth is better at 7°C than at 1, 3 or 5°C.

The survival of both log and stationary phase cells of *S. typhimurium* 13311 in 10% TSB was better at 4°C than at 7°C, as shown in Figures 2 and 6. The reverse is true for log phase cells stored in TSB. For stationary phase cells in TSB there are no differences, as shown in Figures 2 and 6. The formation of filaments by the chilled cells at 7°C might help to explain these results. In the more hypotonic environment of 10% TSB, the filamentous cells may be unable to maintain their integrity and as a result die (i.e. they may be more likely to burst). Filaments in TSB would likely be exposed to less movement of water into the cell, because of the composition of the broth, and would be less likely to lyse.

Resuscitation can be demonstrated by increases in the number of cells able to grow on selective media with no increase in the number of cells able to grow on non-selective media (Stiles *et al.*, 1973) implying that the injured cells have returned to a physiological state comparable with that of the untreated culture. In Figure 5, resuscitation curves for injured log phase cells in TSB and 10% TSB are shown. The more extensively damaged cells that were stored in 10% TSB (a three log cycle difference) could repair themselves in 10% TSB, but could not initiate growth for >5 h. The less injured cells, those stored in TSB (one log cycle difference in plate counts) could repair the injury and initiate growth in *ca.* 2 h. The degree of recovery achieved by any resuscitation treatment depends markedly on the composition of the medium and the conditions of inoculation and incubation (Mossel and Van Netten, 1984).

Moss and Speck (1966) determined that freeze-injured cells of *E. coli* resuscitated when trypticase was added to the resuscitation medium. In this study, a ten-fold dilution of the trypticase soy broth did not increase the time necessary for resuscitation, but it did increase the time necessary for initiation of growth of the microorganisms because:

1. The degree of injury of the cells that were stored in the different broths is dramatically different.
2. The recovery menstruum contained 1/10 of the nutrients in TSB.

It would be more justifiable to compare the resuscitation curves of bacteria stored in the same broth under the same temperature conditions than those stored in different broths. It might be reasonable to say that the extent of injury for cells in the same broth could be estimated by the length of time for resuscitation and growth. Log phase cells stored in 10% TSB at 4°C resuscitated in 2 h at 37°C but did not grow until after 5 h, as shown in Figure 5. When log phase cells were stored in the same broth at 7°C, resuscitation and growth of the chilled culture occurred within 3 h at 37°C, as shown in Figure 8. The viability on TSA of the log phase cells stored in 10% TSB at 7°C was lower than that of log phase cells in 10% TSB at 4°C or stationary phase cells in 10% TSB at 7°C. The more rapid initiation of growth may in part be due to the generation of rod forms of *S. typhimurium* from the elongated cells present in the storage broth.

Microscopic examination of gram stained preparations of the resuscitated cells revealed that the cells were almost entirely short rods. This observation is in accordance with those of Shaw (1968) and Hsu (1972). Shaw (1968) found that six hours incubation at 30°C, was necessary for reversal of filament formation in *E. coli* ML30. Hsu (1972) studied low-temperature induced filaments *S. typhimurium* 13311 and found that, following transfer to 37°C, division of filaments into normal rods occurred rapidly and was virtually complete within 4 h. Hsu (1972) reported that

low-temperature induced filament formation in *S. typhimurium* 13311 could be reversed, as shown by increased plate counts for about 30 minutes in the presence of inhibitors of cell wall and protein synthesis. However, following initial increases, a rapid decline in viable cells occurred. She concluded that the "factor" necessary for the formation of the septum and separation of the cells is made at low temperatures, but that either its activity or some assembly process is blocked at low temperature.

It may be possible to use 10% TSB for resuscitation of injured cells without growth. This would decrease the likelihood that liquid medium recovery would lead to erroneously high estimates of the bacterial content of foods. This is not a problem with the detection of *Salmonella* where a "zero tolerance" level is required for many food samples, but for quantitative counts (e.g. for coliforms) it is desirable to have a resuscitation medium that does not support growth.

For storage of *S. typhimurium* 13311 on chicken meat, the chicken was irradiated with a dose of about 750 krads. At this dosage the residual products of the irradiation should not interfere with the growth/injury studies. Dickson and Maxcy (1984) found that test cultures of *E. coli*, *Pediococcus cerevisiae*, *Moraxella*, *Acinetobacter* and *Micrococcus* spp. were not inhibited on meat which had been irradiated with 1,500 krad.

Fresh meats are frequently contaminated with spoilage organisms such as *Pseudomonas*, *Acinetobacter*, *Brochothrix*, *Alteromonas*, and *Lactobacillus* spp., as well as potential pathogens belonging to the *Enterobacteriaceae* (Gill, 1986) in addition to known pathogens such as *Clostridium perfringens*, *Staphylococcus aureus* and *Campylobacter* spp. (Grau, 1986). As temperatures are lowered, a selective action is exerted on the mixed flora of mesophiles and psychrotrophs (Kraft, 1986). Psychrotrophic bacteria are favored when meat is held at refrigeration temperatures between 0° to 10°C; they grow to large numbers within 1 to 2 weeks, causing spoilage (Kraft, 1986). Since most pathogens will not grow below 5°C, the main problems associated with refrigerated poultry involve the growth of spoilage bacteria (Gill, 1986).

Gill and Newton (1980) found that the growth of *S. typhimurium* and *E. coli* on meat was unaffected by competition with high numbers of naturally occurring meat psychrotrophs such as *Pseudomonas* and *Enterobacter* spp. in aerobically packaged meat. However, when grown on vacuum-packaged meats, the lactobacilli that developed after chilling inhibited the salmonellae. Thus, despite the fact that the background microflora was killed by the γ-irradiation, the growth response of *S. typhimurium* observed in this study can be assumed to be similar to that on fresh chilled meat. However, conditions would be considered more suited to the growth of *S. typhimurium*

because of the reduced numbers of competing organisms. In addition to competing microorganisms, many foods are known to contain antimicrobial substances (Davidson *et al.*, 1983). Although Angelotti *et al.* (1961) reported growth of salmonellae at 6.7°C, most reports of minimum temperatures for growth on foods are higher than that, for example on ground pork, 10°C (Alford and Palumbo, 1969); on ground beef, 12.5°C (Goepfert and Kim, 1975); and on crabmeat and fish, 8°C (Matches and Liston, 1968a). Generally, the minimum growth temperature of salmonellae is lower in agar or broth media than in foods (Mackey *et al.*, 1980b).

In the present study, *S. typhimurium* inoculated onto irradiated chicken meat grew at 7°C. The chicken meat provides an environment that may be richer in nutrients and more protective than the TSB. The low temperature growth of *S. typhimurium* was not "growth" in the conventional sense since the organisms became elongated, even though the plate counts on TSA, BGS, BSA and DA increased. It should be noted that growth at 7°C was observed on irradiated chicken, i.e. in the absence of competing microorganisms.

S. typhimurium 13311 did not grow at 4°C on irradiated chicken meat. This supports the recommendations of many workers that foods be stored at 5°C or below to prevent growth of salmonellae (Prescott and Geer, 1936; Matches and Liston, 1972a). *S. typhimurium* was not injured for growth on the selective media during chilling at 4°C on chicken. Since meat is known to contain cryoprotective compounds such as

proteins, simple and complex carbohydrates and triglycerides (Mackey *et al.* 1980a), it appears that salmonellae on chilled meats are in a relatively healthy physiological condition.

Salmonellae are often present in foods in very low numbers (Campbell *et al.*, 1983; Andrews, 1985) and may be in an injured state. Special procedures have been developed for their detection. There are five basic steps to culture methods for detecting, isolating and identifying *Salmonella*. These are:

1. Pre-enrichment of the sample in a nutritious, non-selective broth
2. Subsequent enrichment in a selective broth (allowing *Salmonella* to proliferate) while suppressing the growth of competing organisms
3. Isolation of pure cultures of *Salmonella* by streaking onto selective agar
4. Biochemical characterization and presumptive identification of isolates suspected of being *Salmonella* and
5. Definitive serological testing (Andrews, 1985).

A major disadvantage of using the culture method is that it takes at least four days to determine if the sample contains *Salmonella*. The primary functions of pre-enrichment are to rehydrate cells that are dehydrated during processing, to allow injured cells to repair themselves and to allow the cells to multiply (Andrews, 1985).

It has been suggested that pre-enrichment for the detection of *Salmonella* in nonprocessed high-moisture foods, such as meat, might be undesirable, because it may allow the growth of competing microorganisms, thus making the detection of *Salmonella* more difficult (FDA, 1984; Mackey, 1984). From the results of the chilling experiments on chicken meat, it seems that pre-enrichment may not be necessary for detection of *Salmonella* on chicken. The microorganisms were not injured for growth on DA, BGS or BSA, so it seems likely that they would be able to withstand the stress of direct selective enrichment. For fresh chicken carcasses, no significant difference was observed in the number of *Salmonella*-positive samples that were pre-enriched in a non-selective broth or directly enriched in selective enrichment media (Thomason and Dodd, 1976; Cox *et al.*, 1978). Thus, pre-enrichment may not be necessary when examining chilled chicken.

Rapid methods such as DNA-DNA hybridization (Fitts *et al.*, 1983), fluorescent antibody technique (Thomason *et al.*, 1957) and enzyme immunoassays (Swaminathan *et al.*, 1985) have been developed in an attempt to reduce the time necessary to determine the presence or absence of *Salmonella* in foods. The basis for obtaining positive results by the cultural method is primarily selectivity, whereas enzyme immunoassays and DNA-DNA hybridization methods depend on the actual numbers of salmonellae present in the sample (Flowers, 1985). He determined that *ca.* 10^7 cells/mL were

necessary for reliable detection of salmonella by DNA-DNA hybridization and enzyme immunoassay methods. Although salmonellae are frequently found on poultry carcasses, they are usually at numbers of less than 30 cells per carcass when determined by whole carcass rinses (Campbell *et al.*, 1983).

Even though salmonellae may not be in a debilitated state on chicken, they must multiply to be detected by DNA-DNA hybridization and enzyme immunoassays. Pre-enrichment, selective enrichment and post enrichment steps have been recommended for *Salmonella* detection using these techniques (Flowers, 1985). When examining fresh, chilled meats it may be possible to eliminate some of the enrichments, since the microorganisms do not appear stressed. Fitts (1985) stated that it is not necessary to purify colonies before doing DNA-DNA hybridization, as food debris and background flora do not give any "signal" in the hybridization. Thus, either a pre-enrichment or a selective enrichment alone may suffice to attain the necessary population levels for detection.

Filaments of *S. typhimurium* 13311 were found to breakdown into rods following transfer to permissive growth temperature in TSB (Hsu, 1972). This was also observed in this study in 10% TSB. But filament degeneration has not been considered for such cells that have been transferred into selenite cystine or tetrathionate broths. If filaments of *S. typhimurium* could revert to rods in the selective

broths, then the need for pre-enrichment of chilled meat samples containing them would seem unnecessary.

Sawyer and Pestka (1985) suggested that food-service systems contained many procedures that could injure bacteria. Injury could be caused by cooking, hot-holding, chilling or freezing. Following initial preparation, many foods are chilled for a period of hours or days before they are served (Sawyer and Pestka, 1985). Chilled foods might become an excellent (but unhealthy) environment for resuscitation of sublethally injured bacteria, not only because of the protein media involved, but also because of the extended time that it may take to cool a food from 60° to 4°C (Sawyer and Pestka, 1985).

To ensure food safety, cellular injury must be considered when assaying foods for pathogens. Inadequate detection of pathogens may occur if sublethally injured cells are exposed to the selective agents in selective media. Some of the selective agars for detecting salmonellae were used as plating media in this study. These were BGS, BSA and DA. BGS contains brilliant green and sodium sulfapyridine, BSA contains bismuth sulfite and brilliant green and DA contains desoxycholate as selective agents. There are many mechanisms proposed that allow bacteria to be resistant to antimicrobial chemicals including: impermeability to the chemical, destruction or modification of the chemical to an inactive form, increasing the synthesis of the target enzyme in excess of the inhibitor or

by the production of a biochemical pathway bypassing the sensitive site (Smith and Amyes, 1984).

Brilliant green penetrates the cell membrane and gains access to the interior of the cell (Foster and Russell, 1971). The mode of action of triphenylmethane dyes, such as brilliant green, is uncertain but it is suggested that they exert their inhibitory effect by interfering with cellular oxidation processes (Pelczar *et al.*, 1977). However it is not known if any brilliant green-sensitive enzymes exist (Foster and Russell, 1971). Brilliant green inhibits the growth of gram-positive bacteria at concentrations between 1:750,000 and 1:100,000. Up to 10 times this concentration is necessary to inhibit gram-negative microorganisms (Foster and Russell, 1971).

Sulfapyridine is thought to inhibit bacterial growth by interfering with tetrahydrofolic acid biosynthesis (Smith and Amyes, 1984). Sulfapyridine, a structural analogue of p-aminobenzoic acid is an antimetabolite. The cells use it and the normal biosynthetic pathway is blocked. When folate synthesis is arrested, cell growth is inhibited due to the cell's inability to synthesize thymidine, purines and several amino acids (Pratt and Fekety, 1986). Sulfonamides are not specific for a special group of organisms, instead they inhibit a wide variety of microorganisms (Pelczar *et al.*, 1977). Sulfonamides are added to brilliant green agar to inhibit the pseudomonads, thus increasing the specificity of the medium (Forney and Miller, 1985).

Bismuth is inhibitory to the growth of all gram-positive bacteria and many gram-negative bacteria, except the salmonellae (Koneman *et al.*, 1979). In combination with brilliant green in bismuth sulfite agar, a highly selective medium is produced (Koneman *et al.*, 1979). BSA does not depend upon lactose fermenting ability for identification purposes (Harvey and Price, 1979). Heavy metals, such as bismuth act antimicrobialy by combining with cellular proteins and denaturing them (Pelčzar *et al.*, 1977).

Agars containing bile salts have long been used for the isolation of enteric bacteria. The selective agents are ox bile or purified bile salts, such as sodium desoxycholate. The treatment of bacteria with bile salts results in a rapid disorganization of the cytoplasmic membrane (Newton, 1958; Hill, 1967). Gram-positive bacteria are more sensitive to bile salts than gram-negative bacteria. Virtually all salmonellae grow well in the presence of bile salts (Koneman *et al.*, 1979). When gram negative microorganisms are stressed they often become sensitive to some of the components in selective media. It has been suggested that damage to surface structures of gram negative bacteria make them more susceptible to agents by allowing the selective agents easier access to their active sites (Ray, 1983, 1986).

The lipopolysaccharide (LPS) molecules on the outer membrane of gram-negative bacteria are arranged on the cell surface in a specific three-dimensional configuration and

act as a barrier against the passage of many surface-active compounds, hydrolytic enzymes and antibiotics (Ray, 1983). Injury to lipopolysaccharides on the outer membrane of gram negative bacteria has been attributed to conformational changes of the LPS probably due to the loss of divalent cations (Kempler and Ray, 1978; Ray, 1983). Such injury, as a result of chilling, has been suggested as a cause of increased sensitivity to long chain fatty acids (Sheu and Freese, 1973; Fay and Farias, 1976), desoxycholate and bile salts (Tang, 1975).

In this study, it is possible that damage to LPS might explain the decreased counts on the selective media. The injured cells might lose their ability to exclude desoxycholate from the cytoplasmic membrane, and thus become sensitive to the surface active agent. Similarly, increased permeability to brilliant green, sulfapyridine and bismuth might explain the increased sensitivities observed in the chill injured cells. Inability of the cells to destroy the selective agent or increased sensitivity of particular enzymes at low temperatures may also explain the increased sensitivity of the injured cells. Chilled storage of *Salmonella* has been shown to result in increased movement of compounds in and out of cells (Tang, 1975). Thus, it is most likely that chilling induced alterations in permeability result in the increased sensitivity to the selective agents used in this study.

To study the fate of salmonellae on chicken more accurately, contaminated chicken would have to be exposed to the conditions of poultry processing. That is, they would have to be exposed to scalding temperatures and immersion chilling in addition to refrigerated storage. The results of these chicken studies only indicate the effect of chilling salmonellae that have contaminated already processed chicken meat. Also, the effect of the background flora should be considered, to test the assumption that they are inconsequential to salmonellae in aerobically packaged raw chicken. This might best be done using nalidixic acid resistant strains of *Salmonella* enabling the survival of *Salmonella* spp. to be assessed in the presence of a background flora. The formation of filaments by *S. typhimurium* might be examined to try to establish the defective step(s) in cell division, possibly by assaying for the presence/absence or activity of PBP3. The significance of filaments of *Salmonella* from chilled foods might also be further studied, for example, to see if the filaments can form rods in selective enrichment cultures or if pre-enrichment techniques are necessary for their degeneration and possibly most efficient recovery.

The results of this study show that cells of *Salmonella typhimurium* 13311 were killed and showed symptoms of injury following storage at refrigerator temperatures. However, the amount of killing and the extent of injury to the survivors is strongly influenced by the physiological age of the

cells, the composition of the menstruum, the storage temperature, the length of time of cold storage, and, perhaps, the morphology of the cells. Chilled TSB and 10% TSB were more stressful environments than chicken meat. In the poultry meat system injury was not detected. Thus, injury which is observed in broth might not be representative of what happens in foods.

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